



UNIVERSITY OF  
LIVERPOOL

# **Hormone Responsive Genes Involved in Osteoporosis in Post- Menopausal Women**

THESIS SUBMITTED IN ACCORDANCE WITH THE  
REQUIREMENTS OF THE UNIVERSITY OF LIVERPOOL  
FOR THE DEGREE OF DOCTOR IN PHILOSOPHY

**By**

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## **DOCTOR OF PHILOSOPHY DECLARATION**

I hereby declare that the research reported in this thesis represents my own work at the Unit of Clinical Chemistry in the Department of Musculoskeletal Biology II (Medicine), Institute of Aging and Chronic Diseases, University of Liverpool during my time between November 2013 and October 2017. All experiments presented in the results section were performed by myself with the exception of 3.2.4.1 and 3.2.4.2 of Chapter 3 which was done by The Genome Analysis Centre (TGAC) (Norwich, UK). This work has not been previously submitted to the university or any other institution in application for admission to a degree or other qualification. The thesis was written by myself with guidance from my supervisors

**Ayed A. Dera** BSc, MSc

## **DEDICATION**

I dedicate this work to my dear parents and my beloved wife as well as my brother and sisters. You have always been there as a source of encouragement and always supported me throughout my life. I hope I have made you proud.

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## List of Abbreviations

Abbreviation	Description
<b>Delta</b>	$\Delta$
<b>3'UHR</b>	3' untranslated region
<b>ADSCs</b>	Adipose tissue derived stem cells
<b>AUC</b>	Area under the curve
<b>ALP</b>	Alkaline phosphatase
<b>BMUs</b>	Basic multicellular units
<b>BP</b>	Biological process
<b>BMMs</b>	Bone marrow macrophages
<b>BMSCs</b>	Bone marrow mesenchymal stem cells
<b>BMD</b>	Bone mineral density
<b>cDNA</b>	Complementary deoxyribonucleic acid
<b>CTSX</b>	Cathepsin K
<b>CTSZ</b>	Cathepsin Z
<b>CSF1R</b>	Colony stimulating factor 1 receptor
<b>DDK1</b>	Dickkopf-1
<b>DNA</b>	Deoxyribonucleic acid
<b>DMSO</b>	Dimethyl sulfoxide
<b>DXA</b>	Dual-energy X-ray
<b>DMEM</b>	Dulbecco's modified eagle's medium
<b>DPBS</b>	Dulbecco's phosphate buffered saline
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>E2</b>	Estradiol
<b>ER<math>\alpha</math>, ER<math>\beta</math></b>	Estrogen receptor $\alpha$ and/or $\beta$
<b>EU</b>	Europe
<b>ECM</b>	Extracellular matrix
<b>FDR</b>	False discovery rate
<b>FBS</b>	Fetal bovine serum
<b>FGF</b>	Fibroblastic growth factor
<b>GO</b>	Gene ontology
<b>GWAS</b>	Genome-wide association study
<b>GH</b>	Growth hormone
<b>HSPCs</b>	Hematopoietic stem and progenitor cells to osteoblasts
<b>HR-pQCT</b>	High resolution peripheral quantitative computed tomography
<b>HRT</b>	Hormone replacement therapy
<b>HRP</b>	Horseradish peroxidase
<b>hASCs</b>	Human adipose-derived stem cells
<b>hFOB</b>	Human fetal osteoblasts
<b>HCC cells</b>	Human hepatocellular carcinoma cell line
<b>IGF-I and II</b>	Insulin-like growth factor I and II

<b>Abbreviation</b>	<b>Description</b>
<b>IL-6</b>	Interleukin 6
<b>LRP5</b>	LDL receptor related protein 5
<b>MCF-7</b>	Michigan Cancer Foundation-7
<b>M-CSF</b>	Macrophage colony-stimulating factor
<b>MSCs</b>	Mesenchymal stem cells
<b>miRNAs</b>	MicroRNAs
<b>mRNA</b>	Messenger RNA
<b>MEF</b>	Mouse embryonic fibroblasts
<b>MDSCs</b>	Myeloid-derived suppressor cells
<b>MDSCs</b>	Myeloid-derived suppressor cells
<b>NHDF</b>	Neonatal normal human dermal fibroblasts
<b>NRT</b>	No reverse transcription control
<b>OA</b>	Osteoarthritis
<b>OC</b>	Osteocalcin
<b>OPN</b>	Osteopontin
<b>OPG</b>	Osteoprotegerin
<b>PBMCs</b>	Peripheral-blood monocytes cells
<b>PDGF</b>	Platelet derived growth factor
<b>PVDF</b>	polyvinylidene difluoride
<b>QC</b>	Quality control
<b>RT-qPCR</b>	Real-time quantitative PCR
<b>ROC</b>	Receiver operating characteristic
<b>RANK</b>	Receptor Activator for Nuclear Factor k B
<b>RANKL</b>	Receptor Activator for Nuclear Factor k B Ligand
<b>RT</b>	Reverse transcriptase
<b>RISK</b>	RNA-induced silencing complex
<b>RPMI</b>	Roswell park memorial institute
<b>RUNX2</b>	Runt-related transcription factor 2
<b>SOST</b>	Sclerostin
<b>SNPs</b>	Single-nucleotide polymorphism
<b>siRNA</b>	Small-interfering RNA
<b>SDS-PAGE</b>	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
<b>SD</b>	Standard division
<b>TGAC</b>	The Genome Analysis Center <sup>TM</sup>
<b>TGF-β</b>	Transforming growth factor beta
<b>TNF</b>	Tumour necrosis factor
<b>TBST</b>	Tris-buffered saline- TWEEN 20
<b>UHR</b>	Ultra-High Recovery
<b>USSCs</b>	Unrestricted somatic stem cells
<b>EGF</b>	Vascular endothelial growth factor
<b>WB</b>	Western blotting
<b>WHO</b>	World health organization

## List of manuscripts, abstract and presentations

**Ayed Dera**, Roger Barraclough, Lakshminarayan Ranganath, Abdullah Y. Mandourah, Dong L. Barraclough. RNA Sequencing Revealed *Estragon Responsive Genes Involved in osteosarcoma cell lines*. Manuscript is written

**Ayed Dera**, Roger Barraclough, Lakshminarayan Ranganath, Abdullah Y. Mandourah, Dong L. Barraclough. *The Role of CTSZ in PBMCs and Osteosarcoma Cell Line*. Manuscript is written

Abdullah Y. Mandourah, Lakshminarayan Ranganath, Roger Barraclough, Sobhan Vinjamuri, Robert Van'T Hof, Sandra Hamill, Gabriela Czanner, **Ayed A. Dera**, Duolao Wang<sup>5</sup>, Dong L. Barraclough *Circulating microRNAs as potential diagnostic biomarkers for osteoporosis*. Paper in progress

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**Ayed Dera**, Roger Barraclough, Lakshminarayan Ranganath, Dong L. Barraclough. *Hormone and Hormone Responsive Stress-related Molecules Involved in Osteoporosis in Post-Menopausal Women*. A poster presentation in 43rd Annual European Calcified Tissue Society Congress (14 May – 17 May 2015, Rome, Italy).

**Ayed Dera**, Roger Barraclough, Lakshminarayan Ranganath, Dong L. Barraclough. *Hormone and Hormone Responsive Stress-related Molecules Involved in Osteoporosis in Post-Menopausal Women*. A poster presentation in Saudi Student conference (31 January – 1 February 2015, London, UK).

# Abstract

The health of our bones is maintained by two major cell types in a delicate balance of active bone remodelling through bone resorption by osteoclasts and bone formation by osteoblasts. An imbalance in the processes leads to osteoporosis, which is a metabolic disorder of the bones that shows increased incidence in post-menopausal women, where estrogen deficiency plays a significant role in its development. Hormone and hormone responsive molecules have emerged as playing a critical role in immunity, metabolism and cell differentiation and their activation is associated with many human diseases. The aim of this project is to investigate hormone and hormone responsive molecules associated with the development of osteoporosis in post-menopausal women. Firstly, a hormone-responsive cell model system was established using osteosarcoma [osteoblast-like] cell lines, Mg-63, TE-85 and Saos-2, which represent three different stages of bone maturation. Secondly, the effect of steroid hormones on these cultured cells was analysed using RNA sequencing to identify differentially-expressed hormone responsive genes. Thirdly, the identified genes were further analysed in peripheral blood mononuclear cells (PBMCs), an osteoclast precursor, from osteoporosis patients, female >40 years, to assess potential diagnostic values for osteoporosis. Furthermore, some functions of selected genes and their protein products were investigated using cultured cells.

A panel of differentially expressed hormone responsive genes was identified using RNA sequencing from established osteosarcoma cell line, Mg-63 after  $\beta$ -estradiol treatment. 154 genes were up-regulated and 108 genes were down-regulated compared to non-treated controls. Further analysis showed that 5 differentially expressed genes were associated with the development of osteoporosis in clinical samples using RT-qPCR.

The levels of annexin A1 (ANXA1), calcium binding protein (S100A4), and X-box binding protein transcription factor (XBP1) mRNAs were significantly decreased in PBMCs associated with reduced T-score in osteoporosis patients compared to osteopenia and non-osteoporotic participants ( $p = 0.0291$ ,  $p=0.00034$ ,  $p=0.0001$ , respectively). Increasing levels of Cathepsin Z (CTSZ), translocation associated membrane protein 2 (TRAM2), and Gasdermin D (GSDMD) mRNAs in PBMCs significantly correlated with reduced T-score in osteoporosis patients

compared to osteopenia and non-osteoporotic participants ( $p = 0.0011$ ,  $p=0.0161$ ,  $p=0.0019$  respectively). The levels of microRNAs miR-100-5p and miR-99a-5p were significantly decreased in PBMCs associated with the reduced T-score in osteoporosis patients compared to osteopenia and non-osteoporotic controls ( $p = 0.0002$  and  $p= 0.0270$  respectively). However, the levels of miR-196b-3p, miR-1260 and miR-1290 were significantly increased in PBMCs associated with the reduced T-score in osteoporosis patients compared to osteopenia and non-osteoporotic controls ( $p = <0.0001$ ,  $p=0.0072$  and  $p=0.0004$  respectively).

Furthermore, through a follow-up cellular functional study, suppression of Cathepsin Z (CTSZ) mRNA/protein expression using RNAi significantly inhibited proliferation, migration and invasion of Mg-63 and TE-85 cells.

This is the first report of the presence of cathepsin Z mRNA/protein in osteosarcoma cells and the first demonstration of an association between CTSZ and osteoporosis in PBMCs. The results suggest that CTSZ might be a valuable diagnostic and therapeutic target for osteoporosis in postmenopausal women in the future.



# **Chapter 1 Introduction**

## 1.1 Bone

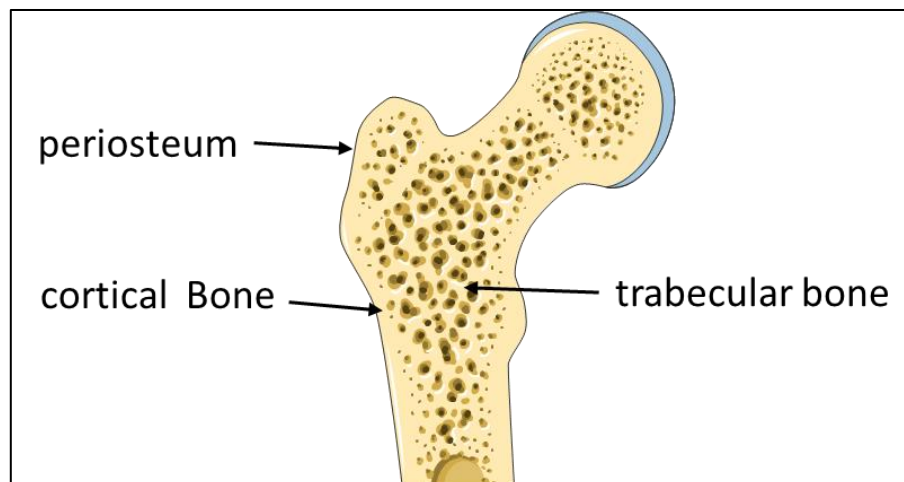
### 1.1.1 Function and Structure of Bone

Bone is a rigid organ that creates the vertebrate skeleton which has a variety of functions. First, it provides structural support to the whole body. Second, it helps in locomotion and movement, through the provision of levers to muscle. Thirdly, bone protects vital structures and internal organs such as the skull protecting the brain or the ribs protecting the heart and lungs. Fourthly, in response to acidic conditions, bone tissue will dissolve to release neutralizing calcium carbonate into the blood stream which eventually maintains the body's proper pH levels. Lastly, bone provides maintenance of mineral homeostasis particularly of calcium and phosphorus (Robling et al., 2006).

There are two types of bone tissue composed of osteons, cortical and trabecular bone, as shown in Figure 1-1. The cortical bone (compact) is dense, solid, and surrounds the marrow space. It comprises 80% of bone in the human skeleton (Clarke, 2008). The trabecular bone (cancellous or spongy) comprises 20% of bone in the human skeleton, and is found sandwiched between cortical bone layers. It is composed of a honeycomb-like network of trabecular plates and rods interspersed in the bone marrow compartment (Clarke, 2008).

Cortical and trabecular bone is comprised of organic and mineral components. The organic component (Osteoid) contains 25% of total bone mass and is composed mostly of cross linked type I collagen (90%), where the rest contains proteoglycan and non-collagenous proteins such as osteocalcin, osteopontin and osteocalcin. The mineral components contain 65% of the bone mass, is mainly calcium and phosphorus in the

form of hydroxyapatite as well as other ions,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{HCO}_3^-$  and the remaining 10% is water. These components provide the rigidity to the structure and the collagen provides resilience and ductility (Florencio-Silva et al., 2015)



**Figure 1-1: Cortical and cancellous bone structure.**

Bone section of the proximal femur in the frontal plane illustrating cortical and trabecular bone. This figure is adapted from ([www.servier.com/Powerpoint-image-bank](http://www.servier.com/Powerpoint-image-bank))

### 1.1.2 Bone Cells

There are four cell types of cell found in bone: osteocytes, osteoblasts, osteoclasts, and lining cells (Downey and Siegel, 2006, Buckwalter et al., 1996).

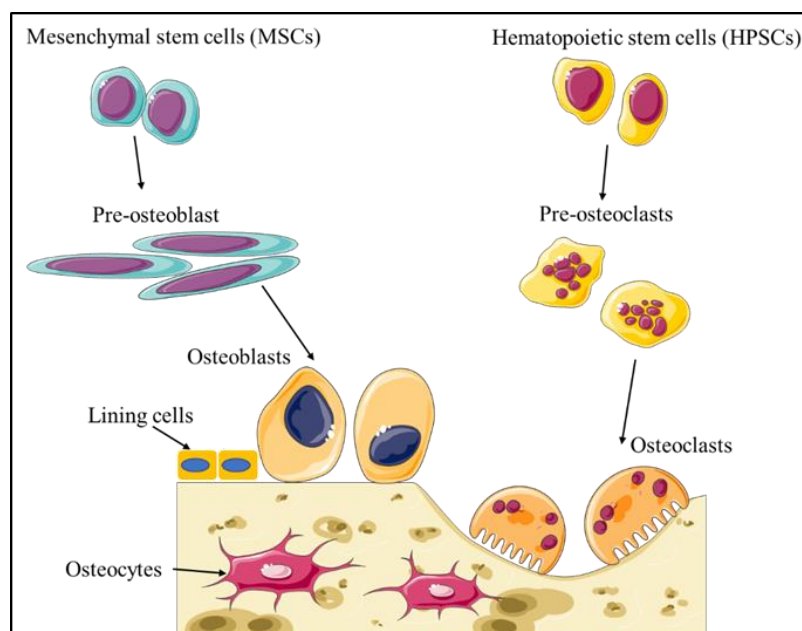
#### 4.2.3.1 Osteoblasts

Osteoblasts are the cells that responsible for bone formation and they are located along the outside of the bone surface (Capulli et al., 2014). They arise when mesenchymal stem cells express the transcription factor RUNX2, a master gene regulator of osteoblast differentiation, forming osteoprogenitor cells or pre-osteoblasts in bone marrow (Figure 1-2). They are found in large numbers in the periosteum, which is a membrane that covers the cortical tissues (Figure 1-1) (Darvin et al., 2013). Osteoblasts are responsible for direct production and secretion of the organic

components of bone, type 1 collagen, and non-collagen protein such as osteocalcin and osteopontin, known as osteoid (Alghazali et al., 2015), which then mineralizes to become bone. On completion of bone formation, the mature osteoblasts can undergo apoptosis (Jilka et al., 1998) or become osteocytes or bone lining cells (Florencio-Silva et al., 2015).

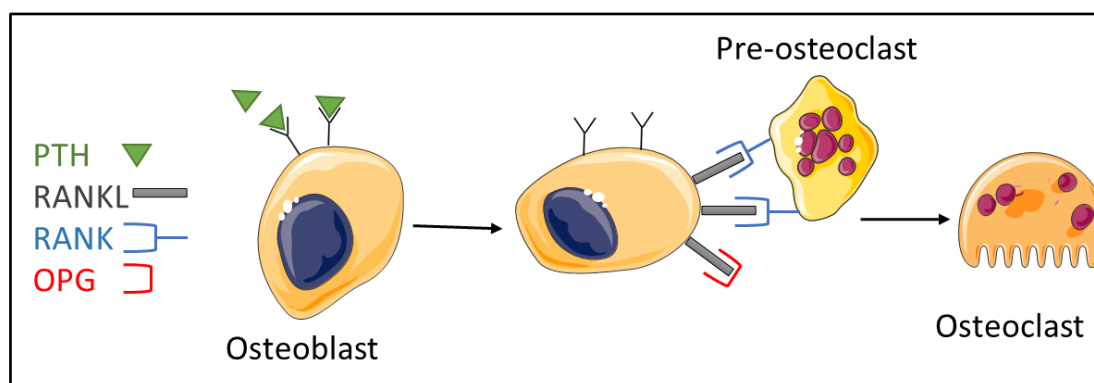
#### **4.2.3.2 Osteoclasts**

Osteoclasts are large cells with multiple nuclei that are responsible for bone resorption (Yavropoulou and Yovos, 2008). Osteoclasts are differentiated from hematopoietic stem cells, the monocyte–macrophage lineage in bone marrow (Boyle et al., 2003), (Figure 1-2.). Bone resorption process is very important in bone health because it allows, along with the osteoblasts, for bone remodelling. The formation of osteoclasts is regulated by cytokines and hormones through several transcription factors, which control osteoclast proliferation, survival, differentiation, and function (Xia et al., 2011). Osteoclast activation occurs on binding of osteoblast membrane-bound Receptor Activator for Nuclear Factor  $\kappa$  B Ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) to their receptors on the surface of osteoclasts (Figure 1-3). RANK signalling is regulated by a decoy receptor for RANKL, osteoprotegerin (OPG), which is produced by osteoblast lineage cells that inhibit the formation of osteoclasts (Lacey et al., 1998), as shown in Figure 1-3. Bone resorption process is controlled by hormones in the bloodstream which instruct the osteoclasts when and where to break down bone tissue through releasing RANKL that activate osteoclasts under the influence of parathyroid hormone (Florencio-Silva et al., 2015, Alghazali et al., 2015)



**Figure 1-2: Schematic diagram of the source of bone cells**

Osteoblasts are derived from MSCs cells that differentiate first to committed pre-osteoblast cells where they form the new bone, and then become osteocyte or lining cells. Osteoclasts originate from HPSCs which differentiate, first to a mononuclear pre-osteoclast cell that then fuses to form the osteoclast cells. This figure is adapted from ([www.servier.com/Powerpoint-image-bank](http://www.servier.com/Powerpoint-image-bank))



**Figure 1-3: Osteoclast activation**

The activation of osteoclast starts from binding of Receptor Activator for Nuclear Factor k B Ligand (RANKL) on osteoblast surface to receptor activator for nuclear factor k B (RANK) on the surface of pre-osteoclast cells. The activation is controlled by osteoprotegerin (OPG) which inhibits the formation of mature osteoclasts by preventing the binding between RANK/RANKL, PTH = parathyroid hormone. This figure is adapted from ([www.servier.com/Powerpoint-image-bank](http://www.servier.com/Powerpoint-image-bank))

### 4.2.3.3 Osteocytes

Osteocytes, which comprise 90–95% of the total number of bone cells, are the most abundant cell type in the bone. They originate from osteoblasts that have migrated into and become trapped inside of the bone matrix (Florencio-Silva et al., 2015). Osteocytes lie within lacunae, within the mineralized bone and have long thin cytoplasmic processes that occupy tiny canals called canaliculi, Figure 1-2. These cells have the ability to form a fine network of connections in the bone matrix with other osteocytes, and with the bone lining cells located at the surface of the bone (Capulli et al., 2014). Therefore, it is thought that these connections are extremely important in cellular communication by facilitating the intercellular transport of small signalling molecules such as prostaglandins and nitric oxide among these cells within the mineralized matrix as well as allowing cell-mediated exchanges of minerals between the fluids in the bone and the vascular supply (Downey and Siegel, 2006, Buckwalter et al., 1996). Recently, however, it has been suggested that osteocytes can alter the rate of bone remodelling by controlling osteoclast formation via production of RANKL (Dallas et al., 2013) and osteocytes also control the balance between formation and resorption of bone by regulating osteoblast formation via production of sclerostin (SOST), which stimulates RANKL expression by osteocytes leading to inhibiting the Wnt signalling pathway (Li et al., 2005) and eventually decreasing the osteoblast formation (Poole et al., 2005, Wijenayaka et al., 2011, Xiong and O'Brien, 2012).

### 4.2.3.4 Lining Cells

This type of cell is derived from osteoblast cells and has an elongated and flattened form (Miller et al., 1989). These cells exhibit a thin and flat nuclear profile and cover the

bone surfaces and separate the bone surface from the bone marrow, thereby protecting bone surfaces from inappropriate resorption (Alghazali et al., 2015), (Figure 1-2). Bone lining cells functions during bone resorption, removing the thin layer of organic component that covers the mineralized matrix once exposed to parathyroid hormone. Also, lining cells prevent the direct interaction between osteoclasts and bone matrix, when bone resorption should not occur, and participate in osteoclast differentiation by producing OPG and RANKL (Alghazali et al., 2015, Everts et al., 2002)

### **1.1.3 Bone Remodelling**

Bone tissue is metabolically active and undergoes continuous remodelling throughout life although it appears to be inert and static (Crockett et al., 2011). Bone remodelling is a process whereby old bone is removed from the skeleton through bone resorption by osteoclasts, and new bone is replaced through bone formation by osteoblasts in a coordinated fashion (Frost, 1990). These processes also could control the reshaping and replacement of bone during growth and following injuries such as fractures and microdamage, which can occur during normal activity. The property of bone remodelling makes the bone a very dynamic tissue that permits the repair of damaged tissue, the maintenance of bone tissue, and the homeostasis of the phosphocalcic metabolism (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b).

#### **4.2.3.1 Bone Remodelling Phases**

At a cellular level, bone remodelling occurs in small areas of the cortical and trabecular surface, known as basic multicellular units (BMUs) (Hauge et al., 2001). The bone remodelling process resorbs the old bone, which is replaced by new bone to prevent accumulation of bone microdamage (Xiao et al., 2016). Under normal conditions, the rate of resorbed bone in the mature skeleton is proportional to rate of production of the newly formed bone (Crockett et al., 2011). Therefore, it is referred to as a balanced process and continues up to the third decade of life in humans, when the bone mass is at its maximum, and this is maintained with small variations until the age of ~50 in humans. From then on, resorption predominates, causing a decrease in the bone mass (Clarke, 2008).



The bone remodelling cycle involves sequential phases that depend on the interactions of osteoclasts and osteoblasts. The process occurs over several weeks and approximately 5 to 10% of total bone is renewed per year (Alghazali et al., 2015). Bone remodelling can be divided into the following six phases, namely, quiescent, activation, resorption, reversal, formation, and mineralization (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b), as summarized in Figure 1-4.

#### **1.1.3.1.1 Quiescent Phase**

This state describes the bone when at rest. The factors that initiate the remodelling process remain unknown (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b).

#### **1.1.3.1.2 Activation Phase**

The bone surface is activated prior to resorption, through the retraction of the bone lining cells and the digestion of the endosteal membrane by collagenase action (Teitelbaum, 2007). The activation stage involves recruitment and activation of osteoclast precursor cells from the circulation, mononuclear monocyte-macrophages, leading to interaction of osteoclast and osteoblast precursor cells (Bruzzaniti and Baron, 2006). This results in the differentiation, migration, and fusion of the large multinucleated osteoclasts in response to macrophage colony-stimulating factor (M-CSF) and RANKL that is released from osteoblasts (Fixe and Praloran, 1998, Fernandez-Tresguerres-Hernandez-Gil et al., 2006b).

#### **1.1.3.1.3 Bone Resorption Phase**

Osteoclasts start to dissolve the mineral matrix by secreting hydrogen ions that create an acidic microenvironment and decompose the osteoid matrix by secretion of lysosomal enzymes, particularly cathepsin K, making the mineralised component

dissolve. Macrophages complete this process and permit the release of growth factors contained within the matrix, essentially platelet derived growth factor (PDGF), transforming growth factor beta (TGF- $\beta$ ), insulin-like growth factor I and II (IGF-I and II) (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b)

#### **1.1.3.1.4 Reversal Phase**

At this stage, bone resorption shifts to bone formation. Once bone resorption is complete, resorption cavities contain mononuclear cells, including monocytes, osteocytes, and preosteoblasts, recruited to begin new bone formation (Wu et al., 2015).

#### **1.1.3.1.5 Bone Formation Phase**

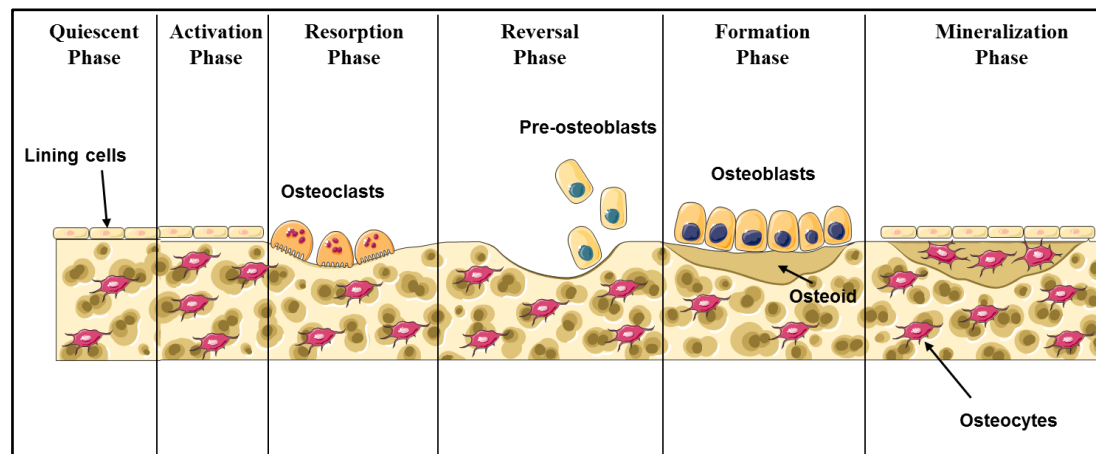
Osteoclasts are replaced by preosteoblast which synthesize a cementing substances, which consists of organic components, type 1 collagen, and non-collagen protein such as osteocalcin and osteopontin, all together known as osteoid (Canalis et al., 2003). A few days later, the resorption cavity is then filled with the osteoid synthesized by the osteoblasts (Lind et al., 1995, Fernandez-Tresguerres-Hernandez-Gil et al., 2006b).

#### **1.1.3.1.6 Mineralization Phase**

This phase begins thirty days after deposition of the osteoid, ending at 90 days in the trabecular, and at 130 days in the cortical bone (Fernandez-Tresguerres-Hernandez-Gil et al., 2006a). Calcium hydroxy phosphate crystals, secreted from osteoblasts, is deposited in the resorption cavity of triple helical fibrils “hole regions” of collagen 1 under the influence of non-collagenous proteins (Gajjeraman et al., 2007, Sapir-Koren and Livshits, 2011). The quiescent phase then begins again after completely cover the newly formed bone surface and link with the osteocytes through a network of canaliculi

in the bone matrix (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b). When the cycle is completed, the amount of bone formed should equal the amount of bone resorbed.

Abnormalities of bone remodelling process are associated with a variety of skeletal disorder such as osteoporosis, which the rate of bone resorption is more than bone formation.



**Figure 1-4: Phases of bone remodelling:**

Bone remodelling requires the coordination of six sequential, but distinct, phases of the process namely, quiescence, activation, resorption, reversal, formation, and mineralization. The quiescent phase involves detection of an initiating remodelling signal, which is described as resorption by osteoclasts. In the resorption phase, osteoblasts respond to signals generated by osteocytes or direct endocrine activation signals, recruiting osteoclast precursors to the remodelling site. The limited duration of the resorption phase depends on the level of the stimulus responsible for osteoclast differentiation and activity. The osteoclasts are replaced by osteoblast in the reversal phase. The formation phase is distinct by forming the osteoid matrix by osteoblastic cells. The mineralization phase includes the crystallization of calcium hydroxy phosphate secreted from osteoblast. This figure is adapted from ([www.servier.com/Powerpoint-image-bank](http://www.servier.com/Powerpoint-image-bank))

### **4.2.3.2 Regulatory Factors of Bone Remodelling**

Some interrelated factors influence the balance between bone resorption and formation processes. These factors are, mechanical, nutritional, local, hormonal and genetic factors (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b).

#### **1.1.3.2.1 Mechanical Factors**

Growing bones are more responsive to physical activity, since it increases bone mineral mass build-up in both children and adolescents (Bonjour et al., 2009). The increased gain in BMD in young athletes compared with less active controls is localized in weight bearing bones. Therefore, in adult athletes, increased bone mass gain from intense physical activity during childhood and adolescence are maintained after training attenuates or even completely ceases in adult athletes (Bonjour et al., 2009). Physical activity influences bone metabolism not only locally, but also systemically (Jacobs et al., 2010). In response to mechanical stimuli early signals produced, include nitric oxide, prostaglandins and IGF-I, which stimulate the osteoblast activity proteins. On the other hand, the absence of physical activity, rest or weightlessness has an adverse effect on bone, accelerating resorption (Jacobs et al., 2010).

#### **1.1.3.2.2 Nutritional Factors**

Generally, the supply of calcium is sufficient to avoid the occurrence of bone disorders during growth (Bonjour et al., 2009). From the last decade, attention has focussed on variations of the intake of certain nutrients that affect bone mass accumulation by healthy children and adolescents (Prentice et al., 2006). Most studies have focused on the intake of calcium to the studies (Moynihan and Holt, 1996, Whitton et al., 2011). Thus, the recommended daily calcium intake, for children aged 6-10 years,

is set at 500 mg, while for female adolescents aged 11-17 it is set at 800 mg in the UK. Nowadays, it is reasonable and safe to recommend food intake that would have about 1000 mg of calcium daily from prepuberty to the end of adolescence, and after that age, and menopause should be at least 1.5 g per day. Similarly, it is known that toxic habits, such as smoking, caffeine, alcohol and excess salt are risk factors for osteopenia, a precursor condition of osteoporosis (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b). Not only an adequate calcium intake is important, but the skin and food supply of vitamin D must be adequate to enhance the mineralization of osteoid and regulate the expression of several bone proteins, particularly osteocalcin (Holick, 1996). Vitamin D expect that bone mass gain can be increased during childhood and adolescence and thus optimal bone mass peak can be achieved (Bonjour et al., 2009). The consequence of reduced level of vitamin D is bone loss, leading to osteoporosis and fractures (Lips and van Schoor, 2011)

#### **1.1.3.2.3 Local Factors**

Bone remodelling is also regulated by local factors. These factors include growth factors, cytokines, and the bone matrix proteins as describe in Table 1-1. They influence in bone remodelling by stimulating bone formation, stimulate bone resorption or inhibit bone resorption. Bone acts as an endocrine organ through the production of prostaglandins and nitric oxide, as well as cytokines and growth factors (Crockett et al., 2011).

**Table 1-1: Regulatory local factors in bone remodelling**

	<b>Stimulate bone formation</b>	<b>Stimulate bone resorption</b>	<b>Inhibit bone resorption</b>
<b>Growth Factors</b>	<ul style="list-style-type: none"> <li>• Bone Morphogenetic Proteins: BMP-2, 4, 6 and 7</li> <li>• Insulin-like growth factor I and II: (IGF-I and IGF -II)</li> <li>• Transforming Growth Factor-<math>\beta</math> (TGF- <math>\beta</math>)</li> <li>• Fibroblastic Growth Factor (FGF)</li> <li>• Platelet-Derived Growth Factor (PDGF)</li> </ul>	<ul style="list-style-type: none"> <li>• Tumor Necrosis Factor (TNF)</li> <li>• Vascular Endothelial Growth Factor (Yue et al.)</li> <li>• Platelet-Derived Growth Factor (PDGF)</li> <li>• Fibroblastic Growth Factor (FGF)</li> <li>• Macrophage-Colony Stimulating Factor (M-CSF)</li> <li>• Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF)</li> </ul>	
<b>Cytokines</b>		<ul style="list-style-type: none"> <li>• Interleukin: IL-1, IL-6, IL-8, IL-11.</li> <li>• Prostaglandins: PGE<sub>2</sub>, PGE<sub>1</sub>, PGG<sub>2</sub>, PGI<sub>2</sub>, PGH<sub>2</sub>.</li> </ul>	➤ Interleukin IL-4

#### 1.1.3.2.4 Hormonal Factors

The endocrine system plays a significant role in the normal development of bone.

The most important hormones that involved in bone physiology are:

##### 1.1.3.2.4.1 Thyroid Hormones

Thyroid Hormones (TH) can stimulate bone resorption and formation by stimulating the synthesis of the osteoid matrix by the osteoblasts and its mineralization. Therefore, in congenital hypothyroidism (cretinism) short stature is produced by the inhibition of the long bone formation. Also, thyroid hormones can stimulate resorption with the increase in number and function of the osteoclasts as bone loss in hyperthyroidism (Kawaguchi et al., 1994).

#### *1.1.3.2.4.2 Parathyroid hormone*

Parathyroid hormone (PTH) produced in the parathyroid glands in response to hypocalcaemia to stimulate the bone resorption by synthesis of RANKL from osteoblast. It controls the homeostasis of calcium by stimulating renal production of 1,25 dihydroxyvitamin D to increase  $\text{Ca}^{+2}$  absorption from kidneys and intestine. Also, PTH stimulates bone formation through the synthesis of IGF-I and TGF- $\beta$  and decreases apoptosis of osteoblasts (Lombardi et al., 2011, Dempster et al., 1993).

#### *1.1.3.2.4.3 Calcitonin*

Calcitonin is produced by the C cells of the thyroid, or parafollicular. It counteracts PTH through inhibiting bone resorption by reducing the number and activity of the osteoclasts (Fernandez-Tresguerres-Hernandez-Gil et al., 2006b). High concentrations of calcitonin might be able to increase urinary excretion of calcium and phosphate, via actions on the kidney tubules (Carney, 1997).

#### *1.1.3.2.4.4 1.25(OH)<sub>2</sub> vitamin D<sub>3</sub> or calcitriol*

Calcitriol is a steroid hormone responsible for increasing the release of calcium and phosphate into the blood from bone by absorption of calcium and phosphate from gastrointestinal tract and kidneys. Thus, it acts in concert with parathyroid hormone (Raisz, 1999).

#### *1.1.3.2.4.5 Insulin*

Insulin stimulates matrix synthesis of insulin like growth factor (IGF-I and II) by the liver and osteoblasts, and found abundantly in the osteoid matrix. They increase the activity of the osteoblasts stimulating collagen synthesis (Guicheux et al., 1998).

#### *1.1.3.2.4.6 Glucocorticoids*

Glucocorticoids increase bone resorption through stimulating osteoclastogenesis by increasing the expression of RANK ligand and decreasing the expression of OPG as well as increasing the apoptosis of mature osteoblasts (Canalis and Delany, 2002).

#### *1.1.3.2.4.7 Growth hormone*

Growth hormone (GH) acts directly on the osteoblasts by with stimulating the activity hormone receptors, thus increasing the synthesis of collagen, osteocalcin and alkaline phosphate (ALP). GH, through an indirectly action, increases in synthesis of IGF-I and II by the osteoblasts (Harvey and Hull, 1997).

#### *1.1.3.2.4.8 Androgens*

Androgens have an anabolic effect by increasing cortical bone size through stimulation of both longitudinal. However, androgen deficiency is associated with lower bone density. Androgens protect men against osteoporosis by maintenance of cancellous bone mass and expansion of cortical bone. Both testosterone and 5 $\alpha$ -dihydrotestosterone stimulate proliferation of osteoblast precursors (Clarke and Khosla, 2009).

#### *1.1.3.2.4.9 Estrogen*

Estrogens play an important role in the regulation of osteoblastogenesis and osteoclastogenesis, by increasing the number and function of the osteoblasts and decreasing the number and function of the osteoclasts in female mice and humans (Imai et al., 2010, Maatta et al., 2013). The decreased levels of estrogen after the menopause plays an important role in the development of osteoporosis in postmenopausal women. The effect of estrogen is mediated by estrogen receptor  $\alpha$  and/or  $\beta$  (ER $\alpha$ , ER $\beta$ ), which have been described in human osteoblasts, osteocytes and osteoclasts (Fitzpatrick,



2006). Estrogen can increase the levels of OPG that inhibits resorption (Hofbauer et al., 1999), so they play an important role in the regulation of osteoclastogenesis. Also, estrogens enhance osteoblast survival and block the osteoblast's synthesis of IL-6 that is a potent stimulator of bone resorption, thus, estrogen deficiency during the menopause is the most important pathogenic factor in bone loss associated with osteoporosis (Nilsson et al., 2001). Estrogen inhibits bone resorption rate in which bone mass decrease and continues to occur years after menopause (Cummings et al., 1998). This would occur through imbalanced production of some cytokines, interleukins and growth factors, which are important in bone development, as will be mentioned in Section 1.2.3.

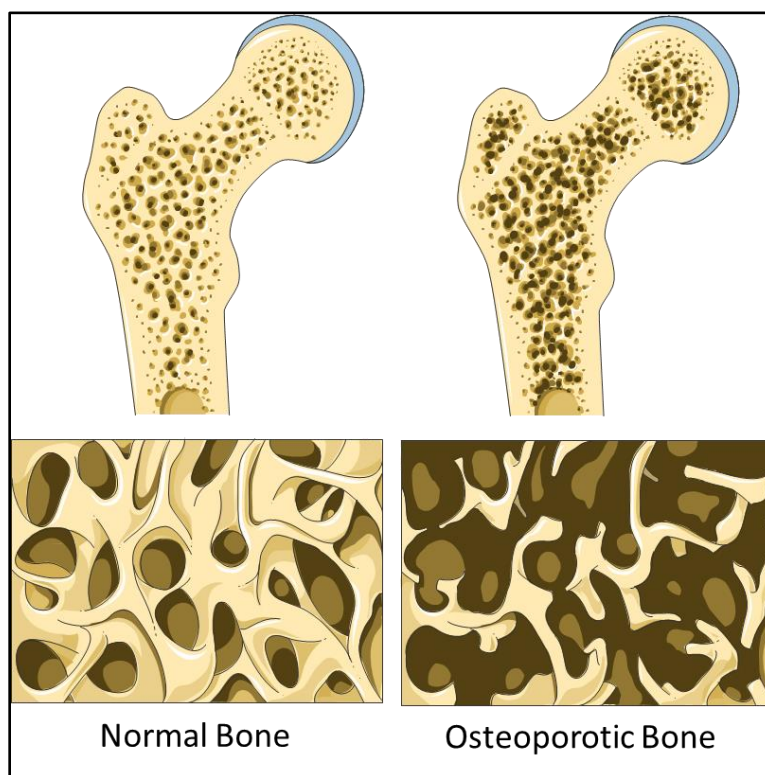
Understanding the basic biology of bone development is important for understanding the molecular and cellular mechanisms underlying bone disorders caused by abnormality in bone remodelling (Table 1-2) (Raisz, 1999, Feng and McDonald, 2011). The bone remodelling process may be derailed at different points leading to various metabolic bone diseases. These diseases, such as osteoporosis, may reflect hereditary or acquired disorders in the mineral phase, the organic matrix, the cellular processes of remodelling, or the endocrine, nutritional, or other factors that regulate skeletal and mineral homeostasis (Xiao et al., 2016). The acquired metabolic bone disorders include: osteoporosis, osteomalacia, the skeletal changes of hyperparathyroidism and chronic renal failure (renal osteodystrophy) and Paget's disease (Feng and McDonald, 2011, Xiao et al., 2016).

**Table 1-2: Abnormalities of bone remodelling in disease**

	<b>Diseases</b>	<b>Bone resorption</b>	<b>Bone formation</b>
<b>1</b>	Primary Osteoporosis • Menopause associated • Age related	increased	increased
<b>2</b>	Secondary Osteoporosis • Glucocorticoid induced • Immobilization induced	increased	decreased
<b>3</b>	Renal osteodystrophy	increased	increased
<b>4</b>	Paget's disease	increased	increased
<b>5</b>	Osteopetrosis	decreased	increased

## 1.2 Osteoporosis

Osteoporosis is a systemic skeletal disorder that characterized by low bone mass, resulting in increased bone fragility and increased fracture risk (Taxel, 1998, Tuck and Francis, 2002), (Figure 1-5). Osteoporosis affects 200 million individuals worldwide and is considered as a silent disease that often remains asymptomatic and undetected and becomes apparent only after bone fracture has occurred (Lin and Lane, 2004). Loss of bone mass and bone strength is mediated by (a) failure to reach the optimal peak bone mass in a young adult, (b) excessive bone resorption, or (c) an impaired bone formation response during bone remodelling (Parra-Torres et al., 2013). Osteoporosis is classified into primary and secondary types. Primary osteoporosis has been divided into two subtypes: failure to reach the optimal peak bone mass in a young adult, (b) excessive bone resorption, or (c) an impaired bone formation response during bone remodelling (Parra-Torres et al., 2013). Osteoporosis is classified into primary and secondary types. Primary osteoporosis has been divided into two subtypes: type I osteoporosis which is a common bone disorder in postmenopausal women caused by estrogen deficiency resulting from menopause or type II osteoporosis, which is associated with aging in both women and men (Feng and McDonald, 2011). Secondary osteoporosis is a group of bone disorders that are complications of various other medical conditions ( Table 1-2), physical activity consequences, or the negative outcome of therapeutic interventions for some disorders (Marcus, 1996).



**Figure 1-5: Difference between normal bone tissue (trabecular) and osteoporotic bone tissue. This figure is redrawn from ([www.servier.com/Powerpoint-image-bank](http://www.servier.com/Powerpoint-image-bank))**

### 1.2.1 Epidemiology of Osteoporosis

Osteoporosis is considered a major public health crisis around the world because it is not diagnosed until the occurrence of fractures, which result in substantial morbidity and mortality (Tuck and Francis, 2002). In 2010, 22 million women and 5.5 million men were estimated to have osteoporosis in Europe (EU), of which 2.5 million women and 680,000 men are in the UK (Hernlund et al., 2013). Fractures caused by osteoporosis affect 1 in 2 women and 1 in 5 men aged >50 years in the UK (Poole and Compston, 2006, Svedbom et al., 2013). 15% of white people of age over 50 and 70% of those over 80 have osteoporosis are affected (Wade et al., 2014). This costs around £3.5 billion annually for health service and treatment with monitoring of fracture patients in the UK (Svedbom et al., 2013). There is an expectation of an increase in cost of treatment of 25

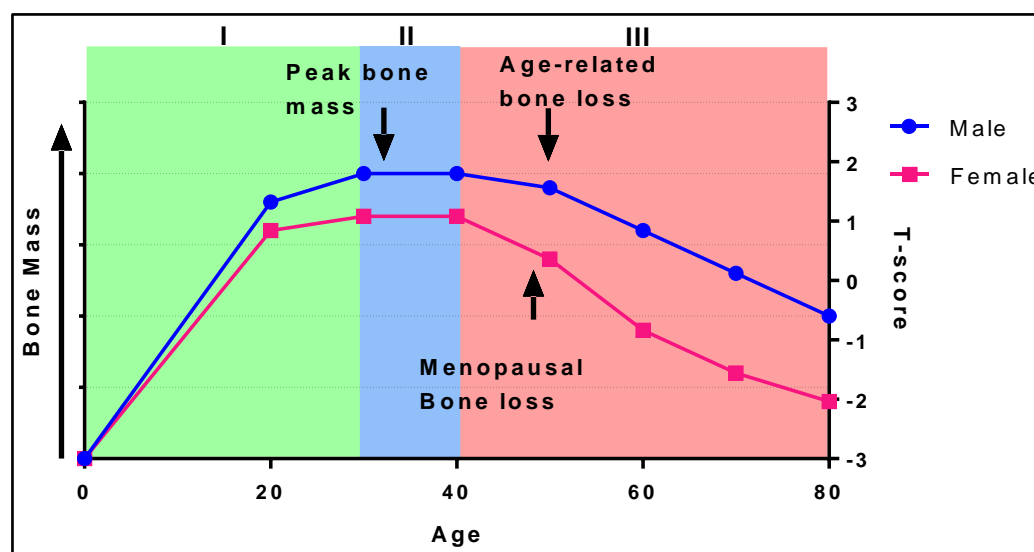
% by 2025 (Hernlund et al., 2013). Svedbom et al (2013) reported that, in 2010, 536,000 new osteoporotic fragility fractures were sustained in the UK, comprising 14.7% hip fractures, 12% vertebral fractures, 12% forearm fractures and 60% other fractures (i.e., fractures of the fibula, clavicle, scapula, rib, humerus, sternum and other femoral fractures).

### **1.2.2 Risk Factors for Osteoporosis**

The most important risk factor for osteoporosis is advanced age in both men and women which is 3-fold higher for each standard deviation (SD) decreased in BMD (Compston et al., 2017). Figure 1-6 shows that women lose bone mass more quickly than men, due to estrogen deficiency following the menopause or surgical removal of the ovaries (Bonjour et al., 1994). In men, a decrease in testosterone levels has a comparable effect (Sinnesael et al., 2013). Race also is a factor where people from European or Asian ancestry are more likely to suffer from osteoporosis than African people (Curtis et al., 2016). Some additional clinical risk factors have been identified. These include: a history of a prior fracture, a parental history of hip fracture, smoking, alcohol intake, malnutrition, and rheumatoid arthritis, which increases fracture risk independently of BMD (Compston et al., 2017).

Genome-wide association studies (GWAS) have proven to be very successful in identifying common genetic variants associated with BMD adjusted for age, gender and weight, however a large portion of the genetic variance for this trait remains unexplained (Liu et al., 2014b, Mori, 2016). Recently, there is evidence to suggest significant genetic correlation between body size traits and BMD and unintended bias can be introduced as a result of adjusting a phenotype for a correlated trait (Mullin et al., 2016). Based on

GWAS data, it was found that there are at least 15 confirmed genes (VDR, ESR1, ESR2, LRP5, LRP4, SOST, GRP177, OPG, RANK, RANKL, COL1A1, SPP1, ITGA1, SP7, and SOX6) and potentially another 30 genes or more that could be osteoporosis related genes (Ralston, 2010). Particularly, these genes are clustered into three biological pathways: the estrogen endocrine pathway, the Wnt/ $\beta$ -catenin signaling pathway and the RANK/RANKL/OPG pathway (Li et al., 2010).



**Figure 1-6: Age-related changes in bone mass in men and women**

The general pattern of bone mass accrual and loss over the three phases of bone development: (I) growth (green), (II) modelling or consolidation (blue), and (III) remodelling (red). This figure is redrawn from (Bonjour et al., 1994)

### 1.2.3 Osteoporosis in Post-menopausal Women

The pathogenesis of postmenopausal osteoporosis is primarily caused by the reduced level of estrogen following the menopause (Feng and McDonald, 2011). Albright et al. (1941), for the first time, demonstrated that estrogen deficiency led to a reduced bone formation in postmenopausal women (Albright et al., 1941). However, subsequent studies (Garnero et al., 1996, Eriksen et al., 1990) indicated that estrogen

deficiency leads to an increase in bone resorption rather than to impaired bone formation. Nevertheless, it is well known that both bone resorption and bone formation are increased in postmenopausal osteoporosis; with the increased of bone resorption rate (Raisz, 2005). The past decades have focused on the understanding of the molecular and cellular mechanisms underlying the role of estrogen deficiency in the pathogenesis of postmenopausal osteoporosis.

Indeed, *in vitro* and *in vivo* studies revealed that estrogen suppresses the expression of IL-1, TNF, and IL-6, which promote osteoclast differentiation, the in monocytes and/or osteoblasts (Pacifici, 1998, Girasole et al., 1992). Pacifici et al (1991) showed that human peripheral-blood monocytes cells (PBMCs) from ovariectomized premenopausal women secrete more IL-1 and TNF (Pacifici et al., 1991). Later Roggia et al (2001) confirmed the pathological role for TNF in postmenopausal osteoporosis by suppressing either TNF or TNF receptor 1 in mice which then showed a resistant to ovariectomy induced bone loss (Roggia et al., 2001). IL-1 and TNF either directly target osteoclasts to enhance its activity or indirectly stimulate osteoclastogenesis by upregulating the expression of IL-6 in osteoblasts (Feng and McDonald, 2011). Overall, these studies confirmed the idea that estrogen exerts protective effects on bone via inhibition of IL-1 and TNF expression. Recent investigations have shown that the increased levels of these three cytokines augment bone resorption by distinct mechanisms.

The RANKL/RANK/OPG system plays an important role in bone remodelling in which any imbalance can lead to bone disorders, including postmenopausal osteoporosis. Osteoblast lineages express both M-CSF and RANKL (Suda et al., 1999), which bind to their receptors, Colony stimulating factor 1 receptor (CSF1R) and RANK,

result in stimulating osteoclast formation (Lacey et al., 1998). RANK regulates osteoclast formation and function in part through recruiting TNF receptor-associated factors (TRAFs), which sequentially regulate osteoclast formation, function, and/or survival (Feng, 2005, Boyle et al., 2003, Takayanagi et al., 2002). OPG, produced from osteoblasts, acts as an antagonist of RANKL functions by competing with RANK for binding of RANKL (Bucay et al., 1998). It has become clear that estrogen exerts bone-sparing effects by targeting the RANKL/RANK/OPG axis through stimulating OPG expression in mouse osteoblasts and stromal cells (Saika et al., 2001). Also, the RANKL expression on osteoblast cells from postmenopausal osteoporosis women was more than that expressed in osteoblast cells from premenopausal controls (Eghbali-Fatourehchi et al., 2003). This indicates that RANKL plays an important role in the pathogenesis of postmenopausal osteoporosis. Furthermore, the RANK-activated JNK pathway, plays a role in cellular apoptosis pathway, in osteoclast cells is inhibited by estrogen (Shevde et al., 2000, Srivastava et al., 2001). As discussed above, deficiency of estrogen results in an increase of IL-1 and TNF secretion, that enhances osteoclastogenesis by increased IL-6 expression. RANKL gene expression in osteoblasts and stromal cells is also stimulated by IL-1 and TNF (Yasuda et al., 1998), which shows the complexity of the action of these two cytokines in the development of postmenopausal osteoporosis.

### **1.2.4 Diagnosis of Osteoporosis**

Dual-energy X-ray (DXA) is the most common technique used for assessing the risk of osteoporosis. It is used to measure hip and spine bone density, and can also measure bone density of the whole skeleton (Kanis et al., 2008). The spine or hip



fracture could be also diagnosis through high resolution peripheral quantitative computed tomography (HR-pQCT) (Nishiyama and Shane, 2013).

The World Health Organization (WHO) has categorised osteoporosis into four groups as shown in Table 1-3. The reference measurement results from bone density measurements in a population of healthy young adults (called a T-score). Osteoporosis is diagnosed when a person's BMD is equal to or more than 2.5 standard deviations below this reference measurement (Kanis, 1994)

**Table 1-3: WHO classification for osteoporosis based on DXA**

Status	BMD
<b>Normal</b>	T-score of -1 or above
<b>Osteopenia</b>	T-score lower than -1 and greater than -2.5
<b>Osteoporosis</b>	T-score of -2.5 or lower
<b>Severe Osteoporosis</b>	T-score of -2.5 or lower, and presence of at least one fragility fracture

There are a few other methods for diagnosing osteoporosis that have been used extensively in clinical trials. These include bone biomarkers.

### 1.2.5 Biomarkers of Bone

Bone biomarkers can be measured in blood or urine. Since bone remodelling processes are coupled and tend to change in parallel, these biomarkers are detected in either in formation and resorption markers that express the metabolic activity of osteoblasts or osteoclasts respectively. Some markers can be used to monitor both processes, e.g. osteocalcin (OC). Also, some of these markers are non-specific for being present in tissues other than bone and may be influenced by non-skeletal processes (Wheater et al., 2013).

### 4.2.3.1 Bone Formation Biomarkers

Bone formation biomarkers are either by-products of active osteoblasts expressed during the various phases of bone remodelling or osteoblastic enzymes. These markers are widely used and measured in serum or plasma and include: bone specific alkaline phosphatase (ALP), osteocalcin (OC) and the carboxy- and amino-terminal propeptides of type 1 collagen (P1CP, P1NP).

#### 1.2.5.1.1 Total alkaline Phosphatase

Alkaline Phosphatase (ALP) is an enzyme found in the bloodstream, which is produced in liver, intestines and kidneys. 50% of total ALP is called bone-specific alkaline phosphatase (BALP) that is a glycoprotein produced from the surface of osteoblasts bone (Masrour Roudsari and Mahjoub, 2012). Abnormal level of ALP isoenzyme in blood indicates an issue with liver, gall bladder, or bones which could be used for diagnosis of bone problems such as rickets, osteomalacia and Paget's disease. The measurement of BALP is also used as an indicator of osteoblastic activity for the assistance of the management of osteoporosis in women (Kuo and Chen, 2017).

#### 1.2.5.1.2 Osteocalcin

Osteocalcin (OC) is synthesized by mature osteoblasts, odontoblasts and hypertrophic chondrocytes (Lee et al., 2007). It plays an important role for metabolic regulation, bone mineralization and calcium ion homeostasis. Serum OC is used as biomarker for osteoblast function for evaluation of bone formation rate in osteoporosis (Kuo and Chen, 2017). The mean level of OC is significantly different between the postmenopausal non-osteoporotic women ( $11.26 \pm 3.07$  ng/ mL) and osteoporotic ( $16.16 \pm 4.5$  ng/ mL) (Singh et al., 2015)

### **1.2.5.1.3 Procollagen type 1 N-terminal and C-terminal Propeptide**

Type 1 collagen included (P1NP& P1CP) can be found in the organic bone matrix, which is synthesized by fibroblasts and osteoblasts. P1CP and P1NP are released during the formation of type 1 collagen (Hassager et al., 1991). P1NP has been demonstrated to be a more sensitive for measuring the bone formation rate in osteoporosis (Garnero, 2008). P1NP has functional advantages where it has low interindividual variability and is relatively stable in serum at room temperature (Wheater et al., 2013).

### **4.2.3.2 Bone Resorption Biomarkers**

Most bone resorption markers are considered degradation products of bone collagen. Earlier research identified bone resorption markers which were measured in urine sample such as pyridinoline (PYD) and deoxypyridinoline (DPD). These biomarkers were time-consuming and cumbersome and relied on second morning void/creatinine ratios, or complete 24 h urine collections, increasing the imprecision of the measurement (Rosano et al., 1998, Lo Cascio et al., 1999). However, now serum/plasma markers are available, such as Osteopontin (OP), Tartrate-resistant acid Phosphatase 5b (TRAP-5b), cathepsin K (CTSX), Carboxy-terminal Crosslinked Telopeptide of type 1 collagen (CTX-1), and Amino-terminal Crosslinked Telopeptide of type 1 collagen (NTX-1) (Wheater et al., 2013)

#### **1.2.5.2.1 Osteopontin**

Osteopontin (OP) is expressed in different cells include bone cells. The plasma level of OP was used to evaluate the treatment of intermittent parathyroid hormone in menopausal osteoporosis (Chiang et al., 2011). Chiang et al found that women with high

level of OP were less resistant to postmenopausal osteoporosis than women with normal OP levels (Chiang et al., 2011)

#### **1.2.5.2.2 Carboxy-terminal Crosslinked Telopeptide of type 1 collagen**

Telopeptides of type 1 collagen, included carboxy-terminal crosslinked (CTX-1) and aminoterminal crosslinked (NTX-1), are used as bone resorption biomarkers (Chiang et al., 2011). They are released during collagen degradation. CTX-1 was found to be a sensitive and specific biomarker of bone resorption that can rapidly indicate the response to bisphosphonate therapy for postmenopausal osteoporosis. However, blood withdrawal must take place in the fasting state because food intake substantially decreases the levels of CTX-1 (Christgau, 2000).

#### **1.2.5.2.3 Cathepsins**

Cathepsins are lysosomal proteases found in all animal cells and have approximately different members of this family, which are distinguished by their structure, catalytic mechanism, and which proteins they cleave (Turk et al., 2012). Cathepsins play a critical role in driving cancer progression, proliferation, invasion and metastasis (Olson and Joyce, 2015), and have ability to catabolize elastin, collagen, and gelatine allow it to break down bone (Brömme and Lecaille, 2009). Some of these cathepsins are expressed highly in specific cell types such as Cathepsin K (CTSK) in osteoclasts and Cathepsin S (CTSS) in immune cells (Mohamed and Sloane, 2006). CTSK plays a role in cancer metastasis through the degradation of the extracellular matrix (Gocheva and Joyce, 2007). The organic bone matrix is degraded by not only cathepsin K, but also by matrix metalloproteinases or other cathepsins (Goto et al., 2003). Each cathepsin is specifically localized in the osteoclast, result in that each cathepsin cooperatively participates to the bone resorption (Goto et al., 2003). CTSK is

an important factor in process of bone resorption where osteoclasts secrete CTSK to degrade the bone matrix proteins included type 1 collagen (Holzer et al., 2005, Troen, 2004). Robust genetic and pharmacological preclinical studies revealed that suppression of CTSK result in increasing the bone mass (Duong le et al., 2016). CTSK level was revealed to be significantly different between non-osteoporotic and osteoporosis patient (Duong le et al., 2016, Holzer et al., 2005) which indicate that CTSK level in the serum could serve as a potential biomarker for fracture prediction and BMD (Kuo and Chen, 2017).

### **4.2.3.3 Bone Turnover Biomarkers**

#### **1.2.5.3.1 Receptor activator of NF- $\kappa$ B ligand**

RANKL is produced by osteoblasts to control the differentiation and maturation of osteoclasts at bone remodelling process. RANKL in serum were studied in fracture risk prediction and evaluation of the response from osteoporosis treatment (Kuo and Chen, 2017). However, more work needs to assess the clinical application of RANKL in osteoporosis diagnosis (Rogers and Eastell, 2005).

#### **1.2.5.3.2 Sclerostin and Dickkopf-1**

Sclerostin (SOST) and Dickkopf-1 (DKK1) are produced by osteoblasts and inhibit the Wnt signalling that responsible for osteoblastic differentiation. DKK1 were positively correlated with the BMD of the femoral neck and of the total hip (Wheater et al., 2013). Also, more clinical trial is needed for using SOST as a biomarker of bone turnover (Kuo and Chen, 2017).

The use of these biomarkers as routine test remains a challenge because of their wide biological and analytical variation (Lewiecki, 2010, Sandhu and Hampson, 2011).

Even with identical assays and methods, the results of different bone markers, including ALP, OC, DPD, and PYD, differed by up to 7.3-fold between 73 laboratories once analysed in two serum and two urine pools (Seibel et al., 2001). The analytical variability is influenced by non-modifiable and modifiable factors. The non-modifiable factor includes: age, sex and ethnicity, geographical location, medication such as glucocorticoids, and Hormone Replacement Therapy (HRT) (Naylor and Eastell, 2012), whereas, the modifiable factors include seasonal changes, phase of menstrual cycle of the patient, level of exercise and the meal intake which found that patients have reduced serum CTX-I levels (20%) 1 hour after breakfast, attributed to the production of the gut hormone, glucagon-like peptide 2 (Henriksen et al., 2003, Naylor and Eastell, 2012). Overall, these bone biomarkers can be used in monitoring treatment efficiency for osteoporosis, but are not useful as a diagnostic tool (Sandhu and Hampson, 2011). Also, although variation of markers was seen, abnormal levels of some of these markers may result from other bone pathologies, not just osteoporosis (Vasikaran and Chubb, 2016)

### **1.2.6 Hormone-Responsive Genes in Osteoporosis**

The accurate and early identification of osteoporosis, commonly in postmenopausal women, would be critical to reduce the physical and economic consequences of this disease, since the current biomarkers, based on normal bone metabolism are ineffective in predicting osteoporosis (Mendoza et al., 2012). Recently, identification of the development of osteoporosis can be achieved through genetic studies since they correlate with the degree of bone mass lost (Mendoza et al., 2012). Thus, the attempt to determine new markers genetically of osteoporosis are necessary because the expression of genes that change during the onset of osteoporosis might be

more effective at detecting the disease in its early stages. However, the identification of genes responsible for the development of osteoporosis is difficult because of the multifactorial nature of the illness, sex and fracture history, and the heterogeneity of different populations, age and ethnicity. To date, more than 150 genes related to osteoporosis have been described, with the three most studied being the vitamin D receptor, estrogen receptors, RANKL and LDL Receptor Related Protein 5 (LRP5) (Mendoza et al., 2012). However, the influence of estrogen deficiency on the pattern of gene expression in the bone of postmenopausal women, which might be associated with the development of osteoporosis, is still unknown.

Estrogen is important in the development of bone by inhibiting bone resorption and inducing bone formation by osteoblasts through its cognate receptors, ER $\alpha$  or ER $\beta$  (Hofbauer et al., 1999, Fitzpatrick, 2006). Table 1-4 shows the genes that are possible targets of hormones in human bone cells or associated with low BMD or osteoporosis, in which some of them were involved in the estradiol pathway (Karasik et al., 2010). For example, single-nucleotide polymorphism (SNPs) of ESR1 were found to be decreased with low BMD, bone remodelling, and fragility fractures (Binh et al., 2006, Langdahl et al., 2000). Moreover, analysis of genes related to the estrogen signalling pathway, Estrogen receptor 2 (ESR2), Bone Morphogenetic Protein 15 (BMP15), and Nuclear receptor interacting protein 1 (NRIP1), more likely to be related to hip osteoporosis (Mendoza et al., 2012). However, Zhao et al showed that interaction of osteoporosis and some genes, such as ESR1 and LRP4 might influence the age at menopause (Zhao et al., 2011). It was found that the interactions between some genes, BMP15, follicle-stimulating hormone receptor (FSHR), Cytochrome P450 Family 19 Subfamily A Member 1 (CYP19A1), and Nuclear Receptor Interacting Protein 1 (NRIP), seem to be

most related to osteoporosis predisposition. However, although osteoporosis has been considered a polygenic disease modulated by environmental factors, the predisposition, pathology and treatment response of the osteoporosis depend on interaction between different genes or between genes and environmental factors. Not only this but also microRNAs are also involved in the pathogenesis of osteoporosis since many reports have recently highlighted the involvement of microRNAs as key regulators of osteoblast mediated bone development, homeostasis and osteoporosis (Lian et al., 2012a).



**Table 1-4: Hormone-related genes in bone cells**

	<b>Hormone</b>	<b>Gene</b>	<b>Function</b>	<b>Reference</b>
<b>1</b>	Estrogen	IGF-1 and -II	Regulate OB differentiation	(Guntur and Rosen, 2013)
<b>2</b>	Estrogen	ESR1	Promote OB differentiation	(Almeida et al., 2013)
<b>3</b>	Estrogen	ESR2	Oppose actions of ESR1, act together with ESR1, or substitute for ESR1 in its absence	(Galea et al., 2013b)
<b>4</b>	Estrogen	RANKL	Stimulate osteoclast differentiation	(Martin et al., 2015)
<b>5</b>	Estrogen	ALP	Increase mineralization of bone	(Scheven et al., 1992)
<b>6</b>	Estrogen	OPG	Inhibits OC differentiation	(Hofbauer et al., 1999)
<b>7</b>	Estrogen	RUNX2	Inhibits OB formation	(Martin et al., 2015)
<b>8</b>	Estrogen	IL-1, IL-6	Stimulate of OC activity	(Nilsson et al., 2001)
<b>9</b>	Estrogen	Cathepsin B, L, K	Stimulate of OC activity	(Pennypacker et al., 2011, Kremer et al., 1995)
<b>10</b>	Estrogen	DKK1	Inhibits osteoblastogenesis, lowers OPG levels, DKK1 enhances RANKL levels	(Pinzone et al., 2009)
<b>11</b>	Estrogen	FasL	Induces OC apoptosis	(Krum et al., 2008)
<b>12</b>	Estrogen	MMP3	Preserved OC differentiation and survival	(Garcia et al., 2013)
<b>13</b>	Estrogen	TNF $\alpha$ , $\beta$ 3	Induced OB apoptosis, inhibits OC differentiation	(Matsuda et al., 2001, Krum, 2011)
<b>14</b>	Estrogen	Bcl2	Induced OB apoptosis	(Pantschenko et al., 2005)
<b>15</b>	Estrogen	SOST	Regulate OB formation	(Galea et al., 2013a)
<b>16</b>	Estrogen	BMP-2, 4, 6	increases OB differentiation	(Zhou et al., 2003, Krum, 2011)
<b>17</b>	Estrogen	C-fos and c-jun	increases OC differentiation	(Oursler et al., 1991, Krum, 2011)
<b>18</b>	Parathyroid hormone	RANKL	Stimulate bone formation	(Dempster et al., 1993)
<b>19</b>	Parathyroid hormone	IGF-I	Decrease OB apoptosis	(Lombardi et al., 2011)
<b>20</b>	Parathyroid hormone	TGF- $\beta$	Decrease osteoblasts apoptosis and decreases bone resorption	(Lombardi et al., 2011)
<b>21</b>	Parathyroid hormone	1.25(OH) $_2$ vitamin D3	Increase Ca absorption from kidneys and intestine	(Lombardi et al., 2011)
<b>22</b>	Insulin	IGF-I and II	Stimulate OB to synthesis collagen	(Guicheux et al., 1998)
<b>23</b>	Glucocorticoids	RANK and OPG	Stimulate of OC activity	(Canalis and Delany, 2002)
<b>24</b>	Growth hormone	IGF-I and II	Increase the synthesis of collagen, osteocalcin and alkaline phosphate	(Harvey and Hull, 1997)

(OB=osteoblast, OC= osteoclast)

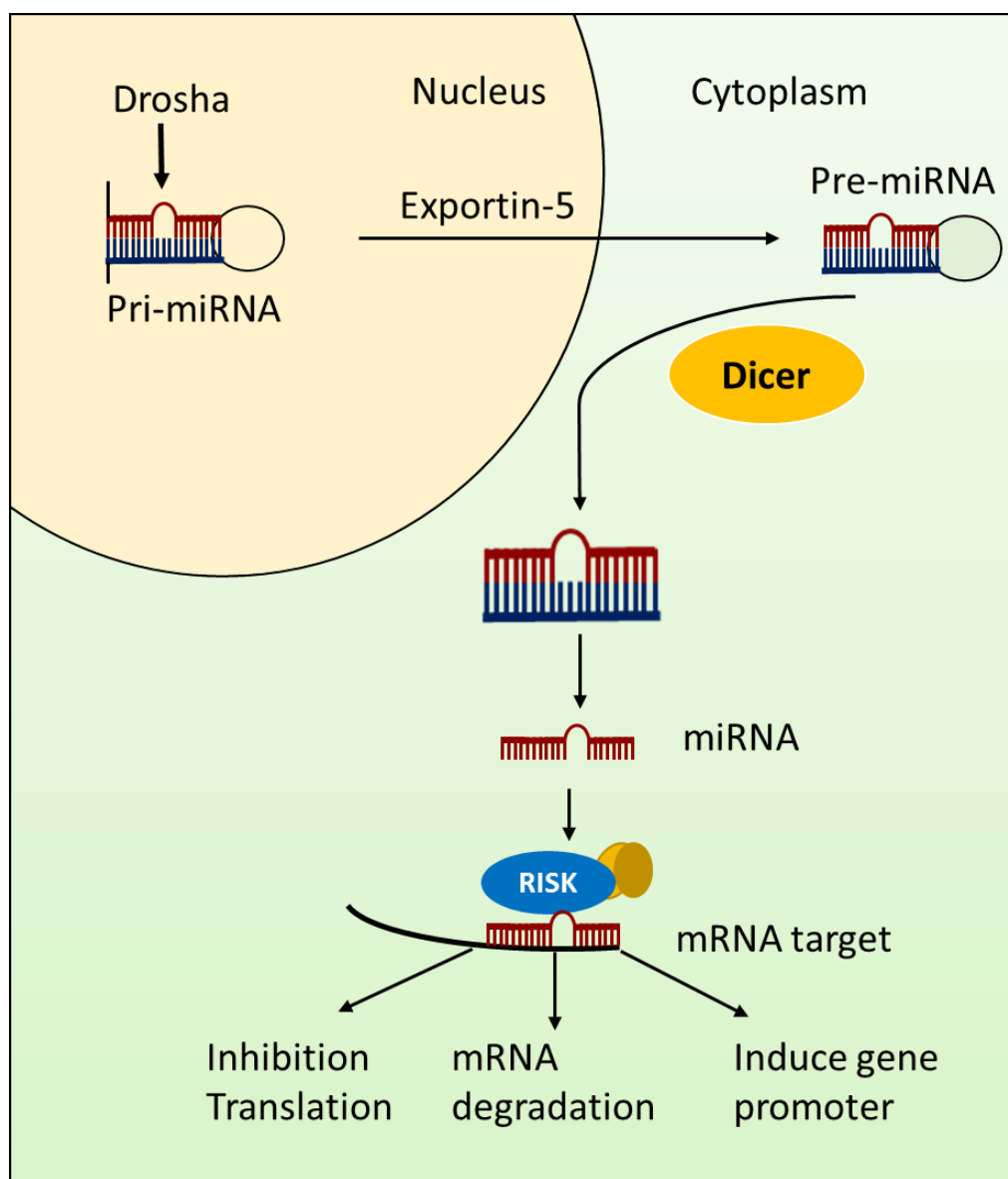
## 1.3 microRNA

microRNAs (miRNAs) are small non-coding RNA molecule containing about 22 nucleotides. miRNAs are involved in signalling pathways, such as control of cellular development, proliferation, and apoptosis (Ambros, 2004, Bartel, 2004). miRNAs function in post-transcriptional regulation of 60% of human protein-coding gene expression through inhibiting the translation of target messenger RNA (mRNA) (Li et al., 2013) or promoting messenger mRNA degradation (Friedman et al., 2009). On the other hand, miRNAs may function to induce gene expression by targeting specific sites in gene promoters (Place et al., 2008). It was demonstrated that miRNAs can activate mRNA translation through the AU-rich element binding protein and conserved miRNA target site in 3' untranslated region (3'UHR) (Vasudevan et al., 2007). The majority of miRNAs are located within the cell, but some have also been found in the extracellular environment or in the circulation (Sohel, 2016). The complexity of miRNA is that a single miRNA can regulate many genes and a single gene can be targeted by different miRNAs (Doench and Sharp, 2004). miRNAs are recognized as major regulators of gene expression, as well as key controllers of different biological and pathological processes (Flynt and Lai, 2008).

### 1.3.1 microRNA Biogenesis

More than 50% of mammalian miRNAs have been found to be encoded within introns of either protein-coding or noncoding transcription units and approximately 10% by exons of long nonprotein-coding transcripts (Ambros, 2004, Bartel, 2004). miRNA genes are transcribed by RNA polymerase II (Lee et al., 2004) or RNA polymerase III (Cai et al., 2004) forming long primary transcripts, pri-miRNAs. Pri-miRNAs are then

cleaved in the nucleus to form hairpin pre-miRNAs (70–100 nt in length) and are transported to the cytoplasm via Exportin-5 for further processing to form double-stranded RNAs of 19–25 nt (Guay and Regazzi, 2013). Pre-miRNAs undergoes cleavage by the endonuclease, Dicer, to form double-stranded RNAs of 19–25 nt (Guay and Regazzi, 2013). One strand is then incorporated into a protein complex called RNA-induced silencing complex (RISC) (Friedman et al., 2009). MiRNA binding can lead to repression of translation or mRNA degradation as shown in Figure 1-7.



**Figure 1-7: microRNA biogenesis**

pri-miRNA is cleaved by Ribonuclease III Drosha enzyme into pre-miRNA in the nucleus and then transported into the cytoplasm by Exportin 5. pre-miRNA is cleaved by Dicer enzyme yielding 21-23 nucleotide duplexes in which one strand binds to RISC and guides translational repression of target mRNAs. This figure is adapted from (Guay and Regazzi, 2013)

### **1.3.2 microRNA Associated with Bone Formation and Bone Resorption**

The roles of miRNAs have been demonstrated by many studies in osteoblasts, osteoclasts, and osteocytes (Moore and Xiao, 2013, Tang et al., 2014, Chen et al., 2015, Ji et al., 2016, Suttamanatwong, 2017). miRNAs are associated with the development, growth, and metabolism of bone and then can cause bone metabolism disorder, resulting in osteoporosis and other bone disease (Bartel, 2004).

### **1.3.3 Regulation of Osteoblast Differentiation by miRNAs**

Many studies revealed that some miRNAs stimulate and others inhibit osteoblastogenesis, and some miRNAs inhibit osteoblastogenesis. Mesenchymal stem cells differentiation to preosteoblast and mature osteoblasts is regulated by runt-related transcription factor 2 (RUNX2) (Zhang et al., 2011b). The differentiation of hMSCs is regulated by miRNAs since Dicer or Drosha knockdown inhibits ALP activity and then decrease the osteogenic differentiation (Oskowitz et al., 2008). A list of miRNAs regulating osteoblastogenesis in human cells is shown in Table 1-5.

**Table 1-5: miRNAs regulating osteoblastogenesis in human cells**

<b>miRNA</b>	<b>Target Gene(s)</b>	<b>Cell Type(s)</b>	<b>Function</b>	<b>Reference</b>
<b>miR-15b</b>	BMPER, PDAP1*, FGF2*	Human MSCs	Stimulate OB Differentiation	(Gao et al., 2011)
<b>miR-20a</b>	PPAR-, Bambi, Crim1	Human MSCs	Stimulate OB Differentiation	(Zhang et al., 2011a)
<b>miR-1228</b>	BMP2K	Human primary osteoblasts	Stimulate OB Differentiation	(Lisse et al., 2013)
<b>miR-29a</b>	DKK1, Kremen2, SFRP	Human primary osteoblasts/FOB 1.19 cells	Stimulate OB Differentiation	(Kapinas et al., 2010)
<b>miR-26a</b>	Smad1	Human ADSCs	Stimulate OB Differentiation	(Luzi et al., 2008)
<b>miR-20a</b>	BMP2*, RUNX2*	Human ligament fibroblasts	Inhibit OB Differentiation	(Zhang et al., 2011a)
<b>miR-31</b>	RUNX2, BMPR2	Human MSCs	Inhibit OB Differentiation	(Gao et al., 2011)
<b>miR-100</b>	BMPR2	Human ADSCs	Inhibit OB Differentiation	(Zeng et al., 2012)
<b>miR-106a</b>	BMP2*, BMPR1A*, BMPR2*, RUNX2*	Human MSCs	Inhibit OB Differentiation	(Gao et al., 2011)
<b>miR-148a</b>	BMP3*, BMP8B*, BMPR2*, RUNX2*	Human MSCs	Inhibit OB Differentiation	(Gao et al., 2011)
<b>miR-221</b>	Wnt signaling molecules	Human USSCs	Inhibit OB Differentiation	(Bakhshandeh et al., 2012)
<b>miR-221</b>	TGF- $\beta$	Human USSCs	Inhibit OB Differentiation	(Bakhshandeh et al., 2012)
<b>miR-1274a</b>	Wnt signaling molecules	Human USSCs	Inhibit OB Differentiation	(Bakhshandeh et al., 2012)
<b>miR-106a</b>	CBFB*	Human MSCs	Inhibit OB Differentiation	(Gao et al., 2011)
<b>miR-138</b>	PTK2	Human MSCs	Inhibit OB Differentiation	(Eskildsen et al., 2011)
<b>miR-146a</b>	IRAK1	Human ADSCs	Inhibit OB Differentiation	(Cho et al., 2010)
<b>miR-1274a</b>	TGF- $\beta$	Human USSCs	Inhibit OB Differentiation	(Bakhshandeh et al., 2012)
<b>miR-424</b>	CBFB*	Human MSCs	Inhibit OB Differentiation	(Gao et al., 2011)

\*Putatively predicted target genes that have not been validated experimentally. (OB = osteoblast)

### 1.3.4 Regulation of Osteoclast Differentiation by miRNAs

There are some miRNAs that regulate the proliferation and differentiation of osteoclasts. The function and effect of miRNA on osteoclast differentiation have been less well studied compared to that in osteoblasts (Franceschetti et al., 2013, Chen et al., 2015). Mammalian miRNAs involved in regulating of osteoclast differentiation are shown in Table 1-6.

**Table 1-6: miRNAs regulating osteoclastogenesis in human cells**

miRNA	Target Gene(s)	Cell Type(s)	Function	References
<b>miR-214</b>	Pten	BMMs	Stimulate OC differentiation	(Zhao et al., 2015)
<b>miR-29</b>	CDC42, SRGAP2	BMMs	Stimulate OC differentiation	(Franceschetti et al., 2013)
<b>miR-21</b>	FasI	BMMs	Stimulate OC differentiation	(Sugatani and Hruska, 2013)
<b>miR-148a</b>	MAFB	PBMCs	Stimulate OC differentiation	(Cheng et al., 2013)
<b>miR-26a</b>	CTGF	BMMs	Inhibit OC differentiation	(Kim et al., 2015a)
<b>miR-34a</b>	TGIF2	BMMs	Inhibit OC differentiation	(Krzyszinski et al., 2014)
<b>miR-7b</b>	DC-STAMP	BMMs	Inhibit OC differentiation	(Dou et al., 2014)
<b>miR-503</b>	RANK	PBMCs	Inhibit OC differentiation	(Chen et al., 2014)
<b>miR-124</b>	NFATcL, RhoA	BMMs	Inhibit OC differentiation	(Lee et al., 2013)
<b>miR-125a</b>	TRAF6	PBMCs	Inhibit OC differentiation	(Guo et al., 2014)
<b>miR-223</b>	NFIA, 1KK $\alpha$	PBMCs	Inhibit OC differentiation	(Shibuya et al., 2013)

(OC= osteoclast)

### 1.3.5 miRNAs as Molecular Biomarkers and Therapeutics for Osteoporosis

Recently, the number of miRNAs associated with osteoporosis has expanded, based on the increasing body of research on miRNAs. However, most of these studies were carried out using cultured cells or animal model systems (van Wijnen et al., 2013), with the exception of a few studies in clinical samples from patients who have osteoporosis fractures in small pilot experiments. MiR-133a is a potential biomarker of osteoporosis and its level in circulating monocytes was significantly increased in 10 osteoporotic postmenopausal Caucasian women compared to 10 non-osteoporotic women (Wang et al., 2012). Seeliger et al (2014) reported that the expression of specific miRNAs (miR-21-5p, miR-23-3p, miR-24-3p, miR-25-3p, miR-100-5p, and miR-125b-5p) in bone tissue significantly increased in osteoporotic compared to that in non-osteoporotic fractures (Seeliger et al., 2014). Also, it was found that 6 miRNAs (miR-133b, miR-328-3p, let-7g-5p, miR-10a-5p, miR-10b-5p and miR-22-3p) in serum from post-menopausal women who had recently suffered osteoporotic fractures at the femoral neck, which were not detectable in control samples (Weilner et al., 2015). However, there is only one study of circulating estrogen related-miRNAs study in osteoclastogenesis and this showed that estrogen down regulates miR-21 in osteoclasts leading to promote the apoptosis is by a FasL mechanism (Sugatani and Hruska, 2013). Thus, it is important to identify differential miRNAs changes relevant to the development of osteoporosis in postmenopausal women who are more susceptible to this disease where estrogen is lost.

RNA sequencing has become quickly a powerful alternative way to measure mRNA expression and uses developed deep-sequencing technologies (Wang et al.,



2009). This technique has several advantages although it is still under active development. First, RNA-seq is not limited to detect transcripts which correspond to existing genomic sequences unlike hybridization-based approaches. This makes RNA-seq able to reveal the precise location of transcription boundaries (Vera et al., 2008). Second, it has very low, if any, background signal because DNA sequences can be unambiguously mapped to unique regions of the genome (Mortazavi et al., 2008). Taking these advantages into account, RNA seq is the accurate method to identify differentially expressed genes in osteosarcoma cells (Mg-63, TE-85, and Saos-2) which represent three different stages of bone maturation treated with Estradiol.

In conclusion, although there are many studies reporting the estrogen-regulation of specific genes in osteoblast cells, and several studies have done on local gene expression in osteoporotic compared to non-osteoporotic bone samples (Krum et al., 2008, Kassem et al., 1996, Chen et al., 2002). However, the link with estrogen deficiency in postmenopausal women is not well understood. This could facilitate understanding of the molecular changes occurring in osteoporosis in post-menopausal women compared to pre-menopausal, by detecting the genes responsible for increasing the risk of osteoporosis once estrogen levels were decreased.

## **1.4 Hypothesis**

The hypothesis underlying this study is that a deficiency of estrogen is responsible for changing genes expression which result in the development of osteoporosis in post-menopausal women.

## 1.5 Aim

The aim of this project is to investigate hormone and hormone responsive molecules associated with the development of osteoporosis post-menopausal women.

1. A hormone responsive cell model system will be established using osteosarcoma [osteoblast-like] cell lines, Mg-63, TE-85 and Saos-2, which represent three different stages of bone maturation (Pacheco-Pantoja et al., 2011).
2. The effect of steroid hormones on these cultured cells will be analysed using RNA sequencing to identify differentially expressed hormone responsive genes.
3. The identified genes will be further analysed in clinical samples from osteoporosis patients to assess potential diagnostic values for osteoporosis.
4. Some functions of selected genes and their protein products will be investigated using cultured cells.

## **Chapter 2 Materials and Methods**

## 2.1 Equipment and Materials

### 2.1.1 Laboratory Equipment Materials

All equipment used in this study are listed in Table 8-1 and all consumable used in this study are listed in in Table 8-2 in the Appendix. Materials for blood collection and molecular biology in this study are listed in Table 8-3 in the Appendix.

### 2.1.2 Antibodies

All antibodies used in this study are listed in Table 2-1 and Table 2-2 with dilutions used for western blotting (Towbin et al.). All species are indicated by the following abbreviations:

**Table 2-1 Primary antibodies used for W.B**

Antibody name	Antigen	Concentration	Company	Cat. number
<b><math>\beta</math>-Actin</b>	Human Actin	1 $\mu$ g/mL	R & D System	MAB8929
<b>ER<math>\alpha</math></b>	Estrogen Receptor alpha	1 $\mu$ g/mL	R & D System	MAB57151
<b>ER<math>\beta</math></b>	Estrogen Receptor beta	2 $\mu$ g/mL	R & D System	MAB7106
<b>CTSZ</b>	Cathepsin Z	1 $\mu$ g/mL	R & D System	AF934

**Table 2-2 Secondary antibodies used for W.B**

Antibody	Species	Antigen	Concentration	Company	Cat. number
<b><math>\alpha</math>-M-HRP</b>	Sheep	Mouse IgG	1 $\mu$ g/mL	Fisher Scientific	10106134
<b><math>\alpha</math>-R-HRP</b>	Donkey	Rabbit IgG	1 $\mu$ g/mL	Fisher Scientific	10710965
<b><math>\alpha</math>-G- HRP</b>	Donkey	Goat IgG	1 $\mu$ g/mL	Fisher Scientific	11596752

### 2.1.3 Primers Used

**Table 2-3 All primers used for RT-qPCR in this study were from QIAGEN**

Accession Number	Gene Symbol	Cat. no.	Band Size	Reference Position	mRNA size
<a href="#">NM 000691.4</a>	ALDH3A1	PPH07009A	166	705	1794
<a href="#">NM 000700.2</a>	ANXA1	PPH02882E	71	395	1399
<a href="#">NM 001001786.2</a>	BLID	PPH57804A	124	444	862

<b>Accession Number</b>	<b>Gene Symbol</b>	<b>Cat. no.</b>	<b>Band Size</b>	<b>Reference Position</b>	<b>mRNA size</b>
<a href="#"><u>NM_004342.6</u></a>	CALD1	PPH21139A	107	1547	4476
<a href="#"><u>NM_001317184.1</u></a>	CDH1	PPH00135F	153	2617	4815
<a href="#"><u>NM_001408.2</u></a>	CELSR2	PPH09555A	110	3925	10540
<a href="#"><u>NM_000088.3</u></a>	COL1A1	PPH01299F	75	3760	5927
<a href="#"><u>NM_000089.3</u></a>	COL1A2	PPH01918B	142	4322	5411
<a href="#"><u>NM_000090.3</u></a>	COL3A1	PPH00439F	95	4287	5490
<a href="#"><u>NM_001336.3</u></a>	CTS2	PPH06136B	72	963	1517
<a href="#"><u>NM_001920.4</u></a>	DCN	PPH01900A	81	1378	2305
<a href="#"><u>NM_001018057.1</u></a>	DKK3	PPH05547F	155	917	2587
<a href="#"><u>NM_001423.2</u></a>	EMP1	PPH16632B	155	2391	2804
<a href="#"><u>NM_001135554.1</u></a>	EPB41L2	PPH20930B	61	2301	3473
<a href="#"><u>NM_000125.3</u></a>	ESR1	PPH01001A	162	245	6330
<a href="#"><u>NM_024939.2</u></a>	ESRP2	PPH18162A	123	2184	4021
<a href="#"><u>NM_000138.4</u></a>	FBN1	PPH06948A	88	8681	11695
<a href="#"><u>NM_021939.3</u></a>	FKBP10	PPH07225A	88	1774	2888
<a href="#"><u>NM_007085.4</u></a>	FSTL1	PPH20210A	108	827	3840
<a href="#"><u>NM_001136007.1</u></a>	FXD3	PPH16470A	189	875	1588
<a href="#"><u>NM_001145453.1</u></a>	GFRA1	PPH01108A	143	1473	9235
<a href="#"><u>NM_014668.3</u></a>	GREB1	PPH20761F	93	1110	8482
<a href="#"><u>NM_001166237.1</u></a>	GSDMD	PPH11430A	143	1030	2326
<a href="#"><u>NM_001206567.1</u></a>	IFI16	PPH01330F	74	2105	2750
<a href="#"><u>NM_001130080.2</u></a>	IFI27	PPH02866B	83	129	681
<a href="#"><u>NM_006820.3</u></a>	IFI44L	PPH05811G	131	424	5889
<a href="#"><u>NM_002038.3</u></a>	IFI6	PPH01322C	63	448	836
<a href="#"><u>NM_001256282.1</u></a>	KRT8	PPH02214F	72	516	1807
<a href="#"><u>NM_001015002.1</u></a>	LLGL2	PPH07188A	139	803	1440
<a href="#"><u>NM_001042544.1</u></a>	LTBP4	PPH10314F	156	4526	5163
<a href="#"><u>NM_052886.2</u></a>	MAL2	PPH10025A	88	2609	2831
<a href="#"><u>NM_001042467.2</u></a>	MLPH	PPH16367B	142	1308	3703
<a href="#"><u>NM_001127891.2</u></a>	MMP2	PPH00151B	69	1452	3416
<a href="#"><u>NM_001144925.2</u></a>	MX1	PPH01325A	184	2542	3444
<a href="#"><u>NM_000909.5</u></a>	NPY1R	PPH01837A	172	1215	2974
<a href="#"><u>NM_001032731.1</u></a>	OAS2	PPH05813A	139	363	2123
<a href="#"><u>NM_032789.3</u></a>	PARP10	PPH17400A	183	1460	3525
<a href="#"><u>NM_001270393.1</u></a>	PKIB	PPH17903B	156	1343	1828
<a href="#"><u>NM_001170690.1</u></a>	SCUBE2	PPH20500B	109	1971	4058
<a href="#"><u>NM_001042422.2</u></a>	SLC16A3	PPH18809E	169	580	2086
<a href="#"><u>NM_001008539.3</u></a>	SLC7A2	PPH00484E	191	7189	7584
<a href="#"><u>NM_004598.3</u></a>	SPOCK1	PPH10509A	128	1166	4841
<a href="#"><u>NM_003222.3</u></a>	TFAP2C	PPH06080A	180	1467	2895
<a href="#"><u>NM_000358.2</u></a>	TGFB1	PPH01904D	191	2480	2805
<a href="#"><u>NM_022152.4</u></a>	TMBIM1	PPH20239B	81	705	2375

Accession Number	Gene Symbol	Cat. no.	Band Size	Reference Position	mRNA size
<a href="#">NM_001008495.3</a>	TMEM64	PPH10400A	67	1017	4819
<a href="#">NM_001003395.1</a>	TPD52L1	PPH08107A	92	406	1409
<a href="#">NM_012288.3</a>	TRAM2	PPH58037A	66	1119	7065
<a href="#">NM_001005207.2</a>	TRIM37	PPH07602A	94	2899	3623
<a href="#">NM_003380.3</a>	VIM	PPH00417F	92	1545	2151
<a href="#">NM_017523.3</a>	XAF1	PPH58211A	172	889	3638
<a href="#">NM_001040275</a>	ESR2	PPH00992C	81	941	2470
<a href="#">NM_020529</a>	NFKBIA	PPH00170F	133	835	1579
<a href="#">NM_000478</a>	ALP	PPH01311F	63	37	2606
<a href="#">NM_002961</a>	S100A4	PPH01313E	57	249	512
<a href="#">NM_005978</a>	S100S2	PPH00423E	113	834	970
<a href="#">NM_001015151</a>	RUNX2	PPH01897C	102	1555	5487
<a href="#">NM_005080</a>	XBP1	PPH02850A	133	740	1820
<a href="#">NM_001256799</a>	GAPDH	PPH0050F	130	856	1455

**Table 2-4 All miRNAs used for RT-qPCR in this study were from QIAGEN**

NO .	miRNA	miRBase microRNA database/Forward Primer Sequence	QIAGEN miScript Cat.
1	hsa-miR-1281	MIMAT0005939: 5'UCGCCUCCUCCUCUCCC	MS00014455
2	hsa-miR-1290	MIMAT0005880: 5'UGGAUUUUUGGAUCAGGGA	MS00014518
3	hsa-miR-143-3p	MIMAT0000435: 5'UGAGAUGAAGCACUGUAGCUC	MS00003514
4	hsa-miR-4516	MIMAT0019053: 5'GGGAGAAGGGUCGGGGC	MS00037555
5	hsa-miR-409-3p	MIMAT0001639: 5' GAAUGUUGCUCGGUGAACCCCU	MS00006895
6	hsa-miR-1260	MIMAT0005911: 5'AUCCACCUCUGCCACCA	MS00014329
7	hsa-let-7c-5p	MIMAT0000064: 5' UGAGGUAGUAGGUUGUAUGGUU	MS00003129
8	hsa-miR-100-5p	MIMAT0000098: 5'AACCCGUAGAUCCGAACUUGUG	MS00003388
9	hsa-miR-450a-5p	MIMAT0001545: 5'UUUUGCGAUGUGUCCUAAUAU	MS00006937
10	hsa-miR-196b-3p	MIMAT0009201: 5'UCGACAGCACGACACUGCCUUC	MS00031570
11	hsa-miR-373-5p	MIMAT0000725: 5'ACUCAAAAUGGGGGCGCUUCC	MS00006867
12	hsa-miR-99a-5p	MIMAT0000097: 5'AACCCGUAGAUCCGAUCUUGUG	MS00003374
13	hsa-miR-145-3p	MIMAT0004601: 5'GGAUUCUGGAAAUACUGUUCU	MS00008708
14	hsa-SNORD96A	CCTGGTGATG ACAGATGGCA TTGTCAGCCA ATCCCCAAGT GGGAGTGAGG ACATGTCCTG CAATTCTGAA GG	MS00033733

## **2.2 Cell Culture**

### **2.2.1 Cell Culture Technique**

Cell culture work was carried out in a sterile cell culture hood class II. Medium was warmed at 37°C in the water bath before use. All flasks were labelled properly, covered loosely, and kept at 37°C in a humidified atmosphere of 95% air, 5% CO<sub>2</sub> incubator. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) foetal bovine serum (FBS), 2 % (v/v) 200 mM L-glutamine, 1 % (v/v) 100IU penicillin/streptomycin and 1 % (v/v) of MEM Non-Essential Amino Acid Solution. This medium is referred to as 'complete medium' in this study. All routine culture media were replaced every alternate day.

### **2.2.2 Cell Lines**

Three types of the osteosarcoma cell lines were used, Mg-63, TE-85, and Saos-2 represent three different stage of maturity (Pacheco-Pantoja et al., 2011, Clover and Gowen, 1994, Rodan et al., 1987) based on measuring alkaline phosphatase activity and osteocalcin production, markers that are representative of early and late stages of osteoblast maturation (Clover and Gowen, 1994). The Mg-63 cell line, which was derived from an osteosarcoma of a 14 years old male, is considered as an undifferentiated initial osteoblast phenotype (Clover and Gowen, 1994, Pacheco-Pantoja et al., 2011, Heremans et al., 1978). The TE-85 cell line, which was derived from a sarcoma of a 13 years old Caucasian female, represents osteoblasts with a higher level of maturity than Mg-63 (Clover and Gowen, 1994, Pacheco-Pantoja et al., 2011, McAllister et al., 1971). The Saos-2 cell line, which was derived from an osteosarcoma

from an 11-year-old Caucasian female, represents the most mature osteoblasts (Pautke et al., 2004, Pacheco-Pantoja et al., 2011). The MCF-7 cell line is a breast cancer cell line isolated in 1970 from a 69- year-old Caucasian woman (Soule et al., 1973). This cell line was used in this study as an estrogen receptor positive control cell line (Levenson and Jordan, 1997).

### **2.2.3 Thawing of Cell Stocks**

Stocks of frozen cells in freezing medium were removed from liquid nitrogen and thawed rapidly at 37°C in a water bath. The cells were immediately transferred to a sterile tube containing 10 mL of complete medium. The tube was centrifuged at 100 g for 5 mins at room temperature in a bench-top centrifuge, after which the cell pellets were resuspended in the appropriate volume of required complete medium for plating at a density of approximately  $2.5 \times 10^4$  cells per  $\text{cm}^2$ . All flasks or plates were then kept as mentioned in 2.2.1.

### **2.2.4 Routine Cell Culture**

All cells were cultured in a humidified atmosphere of 95% air, 5% CO<sub>2</sub> at 37°C in complete medium until about 70-80% confluent. The cells were washed twice with 10 mL Dulbecco's phosphate-buffered saline (PBS), then incubated with 0.01% (w/v) Trypsin-EDTA at 37°C for 2 min until approximately 90% of the cells had detached from the surface of the flask. The trypsin solution was inactivated by adding 10 mL of complete medium. The cells were then transferred into a sterile universal tube and centrifuged at 100 g for 5 minutes. The supernatant was removed, Cells were re-



suspended in the appropriate volume of required medium and replaced at a dilution of 1:4, (see Table 2-5).

**Table 2-5 Routine Cell Culture and Passaging**

Cell	Medium	Ratio	Passage (from-to)
<b>Mg-63</b>	DMEM containing 10% FBS	1:4 every 2 days	P3-P9
<b>TE-85</b>	DMEM containing 10% FBS	1:4 every 2 days	P2-P11
<b>Saos-2</b>	DMEM containing 10% FBS	1:4 every 2 days	P2-P9
<b>MCF-7</b>	DMEM containing 10% FBS	1:4 every 2 days	P10-P18

## 2.2.5 Cell Counting

Cells were counted using a Scepter™ cell counter according to manufacturer's recommendations. Briefly, cell pellets were resuspended and mixed well with 1 mL of PBS in a 2 mL sterile microcentrifuge tube. 50 µl of the cell suspension was drawn into a disposable 60 µm sensor and cell number was calculated.

## 2.2.6 Freezing of Cultured Cells

Cells were selected for freezing when they were approximately 70-80% confluence. The cells were detached as described in 2.2.4 and resuspended in 10 mL of medium containing 10% FBS. The cells were re-centrifuged at 100 g for 5 min. The cells were resuspended in the appropriate volume of freezing medium containing 7.5% (v/v) DMSO to give a concentration of  $1 \times 10^6$  cells/mL. One mL aliquots of cell suspensions were frozen in sterile cryogenic vials in a Nalgene cryo-preserved box containing 250 mL of isopropyl alcohol stored in a  $-80^\circ\text{C}$  freezer overnight, before being transferred to liquid nitrogen for long-term storage.

## **2.2.7 Cell Culture with Hormone-Stripped Serum**

### **2.2.7.1 Preparation of “Steroid hormone-stripped” Serum**

Steroid–stripped serum was prepared from FBS as follow. The FBS was treated with dextran-coated charcoal containing 2% (w/v) of activated charcoal (Sigma, UK) by incubation at 56°C for 45 min initially, then overnight at 4°C with gently rocking. The charcoal was removed by centrifugation at 500 g for 30 min at 4°C. The procedure was then repeated once and the serum finally sterilized by filtration through both 0.45  $\mu$ m and a 0.22  $\mu$ m filter and stored frozen at -80°C.

### **2.2.7.2 Optimization the Concentrations of “Steroid Hormone-Stripped” FBS Medium**

The cells were grown in complete medium until reaching about 60 % confluency and transferred to phenol red-free Roswell Park Memorial Institute Medium (RPMI) supplemented with 0.0, 0.25, 0.5, 1, 2.5, or 5% concentrations of hormone-stripped FBS for 6 days followed by collection of the cells. Medium was changed daily, and cells were washed once with PBS prior to each medium change.

### **2.2.7.3 Culture of Cells in 0.5% “Steroid hormone-stripped” medium**

The cells were grown to 60% confluence, washed twice with PBS and transferred into RPMI containing 0.5% ‘hormone-stripped’ FBS and cultured for 3 days to deplete the endogenous steroids. Medium was changed daily, and cells were washed once with PBS prior to each medium change. Estrodial was added to a final concentration of  $10^{-8}$  M to the medium on the 3<sup>rd</sup> day of being cultured in a stripped medium followed by collection cells after 48h.

## **2.3 Isolation of RNA**

### **2.3.1 Isolation of total cellular RNA**

Total cellular RNA was prepared using a combination of TRIzol® reagent and PureLink® RNA Mini Kit, according to manufacturer's recommendations (Ambion, UK). Generally, for isolation of total cellular RNA, when cells reached 70-80% confluence, they were harvested and washed with PBS, which was removed by centrifuging at 1000 g for 5 min. 1 mL of TRIzol® reagent was added to the cell pellet and mixed with 200 µL of chloroform followed by mixing the tube vigorously and centrifuging at 14,000 g for 30 min. RNA was precipitated by adding an equal volume of 70% ethanol and then aspirated onto a RNA spin cartridge tube. The column was washed with wash buffer I and wash buffer II supplied by the manufacture (Ambion, UK) followed by spin at 8000 g for 15 seconds. The column was transferred to a new 1.5 mL recovery tube, subsequently adding 30 µL pre-warmed RNase-free PCR water directly onto the column to get the 1<sup>st</sup> RNA elution. The resulting purified RNAs were stored frozen at -80°C until use. 1.5 µL of every sample was taken to determine the RNA concentration using a Nano Drop 2000 Spectrophotometer.

### **2.3.2 RNA Extraction from PBMCs Using miRCURY Kit**

Total RNA from clinical samples PBMC was extracted using miRCURY Kit according to manufacturer's recommendations [Exiqon]. PBMC cell pellets were lysed in 350 µL of Lysis solution containing 1% of 2-mercaptoethanol and vortexed for 15 secs followed by the addition of 200 µL of 100% ethanol. The suspension was transferred to a column provided with a collection tube and then centrifuge the column at  $3500 \times g$  for 1

minute. The column was washed twice by 400  $\mu$ l of wash solution and centrifuged for 1 min at 5000 g. RNA was then collected by adding 50  $\mu$ l of elution buffer to the column followed by centrifuged for 2 min at 200 g, followed by 1 min at 14,000 g. 1.5  $\mu$ l of every sample was taken to determine the RNA concentration using a Nano Drop 2000 Spectrophotometer.

### 2.3.3 RNA Concentration and Purity

The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The measurement of RNA concentration was expressed as in ng/ $\mu$ l calculated from absorbance at 260 nm using the modified Beer-Lambert equation (Evans), and the ratio of the absorbance at 260/280 and 260/230 nm ratios were also calculated to assess the purity of nucleic acids (RNA) from either protein or phenol contaminant. While the ratio of the absorbance at 260/230 was used to measure the nucleic acid purity from residual phenol, residual guanidine from the Trizol reagent used for precipitation of the RNA. These ratios are used to assess the purity of RNA, where values of 2 or above are considered as RNA pure for further experimentation (Johnson et al., 2012).

## 2.4 RNA Sequencing

6  $\mu$ g of RNA was sent to The Genome Analysis Centre TM (TGAC) (Norwich, UK) for RNA sequencing in order to identify differentially expressed mRNA between control and treated cells. The quality of RNAs was checked using the Agilent Bioanalyser 2100. Illumina TruSeq RNA libraries from a total of 8 RNA samples were constructed and RNA sequencing of the resulting 8 libraries on a HiSeq2000 DNA

sequencer with a 100bp paired-end read-metric performed by TGAC. Bioinformatics analysis of RNA sequencing was carried out by TGAC including RNA sequencing data QC, filtering and mapping, transcript assembly, normalization and differential expression analysis. Alignment of RNA-Sequencing reads to transcriptome reference [Human genome build 38] was carried out using TopHAt software followed by transcript reconstruction and differential expression of RNA sequencing using Cufflinks software.

## **2.5 Transient Transfection using RNAi Technology**

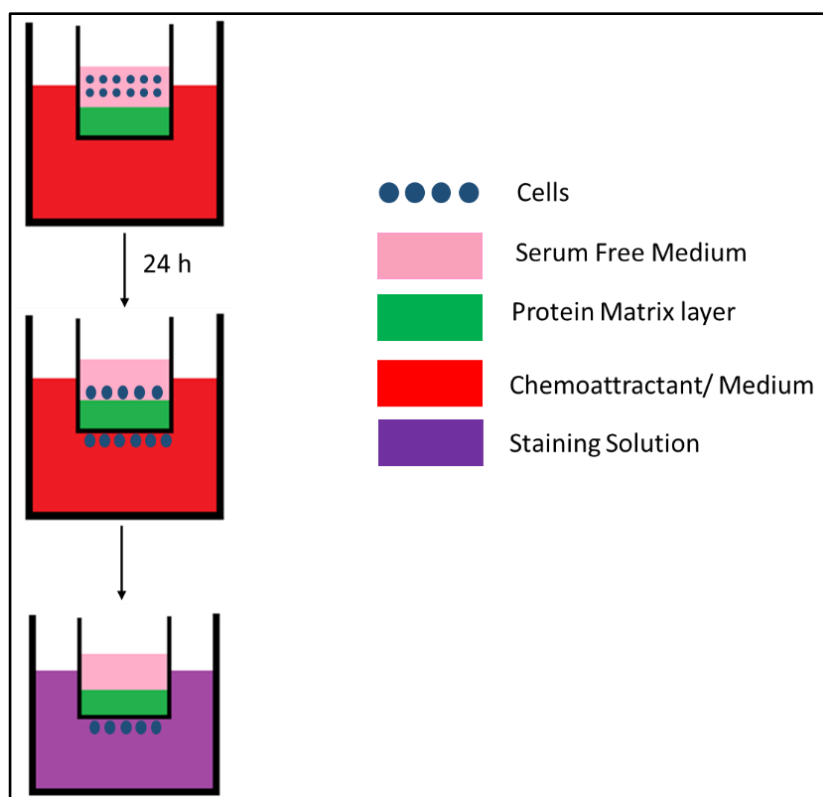
Cells were transiently transfected with siRNA using HiPerFect<sup>®</sup> Transfection Reagent (QIAGEN #1301702). Transient transfections were performed using 6-well cell culture plates. One well contained parental cells, scrambled RNA, transfection reagent only, while cells in another well were treated with the 4 siRNAs. Cells were seeded at  $1 \times 10^5$  per well 24 h before the transfection which was carried out when the cells reached 50-60 % confluence. 1  $\mu$ l of siRNA was diluted in 100  $\mu$ l of medium without serum and 3  $\mu$ l of HiPerFect<sup>®</sup> Transfection Reagent, to give a final concentration of 5 nM in a 2 mL final volume after adding the complexes to cells. The mixture was then mixed by vortexing followed by incubation for 5-10 mins at room temperature to allow the formation of transfection complexes. The medium containing the transfection complexes was added drop-wise onto the cells and the plate gently swirled to ensure uniform distribution of the transfection complexes. Cells were incubated under their normal growth condition and gene silencing was monitored after 24 hr. Cells were then used experimentally to extract RNA or to prepare protein samples.

## 2.6 Cell Invasion Assay

Invasion assays were performed using the (Yue et al.) Matrigel™ Invasion Chamber (BD Biosciences, USA) according to manufacturer's recommendations. Briefly, 0.5 mL of warm culture medium was added to the interior of the inserts and allowed to rehydrate for 2 h in a humidified tissue culture incubator at 37°C, 5% CO<sup>2</sup> atmosphere. After rehydration, medium was carefully removed without disturbing the GFR Matrigel™ Matrix layer on the membrane. 2.5×10<sup>4</sup> cells in 0.5 mL serum free medium were loaded into every upper compartment of chambers while 1 mL of complete medium was placed in the lower compartments of each well, (Figure 2-1). A negative control was set up for each cell line by using serum-free medium in the lower compartment. The matrigel invasion chambers were incubated in a humidified tissue culture incubator for 24 h. Non-invading cells were removed from the upper surface of the membrane by scrubbing using a cotton wool swab. The scrubbing was repeated with a second swab moistened with medium. The cells on the lower surface of the membrane were stained with Shandon™ Kwik-Diff™ Stains by incubating each insert for approximately 30 seconds in each solution followed by washing 2 time with PBS and allowed to air dry for minimum of 60 minutes.

The results were presented as the percentage of cells invaded through the GFR Matrigel™ Matrix™ membrane relative to the control.

$$\% \text{ Invasion} = \frac{\text{Mean \# of cells invading through GFR Matrigel insert membrane}}{\text{Mean \# of cells migrating through control insert membrane}} \times 100$$



**Figure 2-1: Schematic diagram illustrating invasion assay using the (Yue et al.) Matrigel™ Invasion Chamber.**

Cells were seeded in serum free medium and then leaving overnight to penetrate through the membrane follow by staining the membrane and count cells using inverted microscope.

## 2.7 Wound Healing Assay

A scratch wound healing assay was carried out by capturing images at regular intervals by time lapse microscope (Rodriguez et al., 2005, Kramer et al., 2013). Cells were seeded at  $1 \times 10^5$  per well in a 6-wells plate for 24 h and then treated with the transfection complex as mention in section 2.5. After achieving confluence, the cellular layers in all plates were scratched using sterile 200  $\mu$ l pipette tips and rinsed with PBS. Cell migration at the edge of the scratch was analysed at 0, 24, 48, and 72 h. Images were captured using a 4x objective lens of Nikon Diaphot inverted microscope/D50 camera with an ToupTek ToupView software. The width of the wound between the two edges was measured in triplicate using Image J software.

## 2.8 Cell Proliferation Assay

### 2.8.1 Preparation of growth curve

Proliferation assays were carried out using an MTT assay. Parental Mg-63 and TE-85 cells were grown to 70-80% confluence, harvested as previously outlined and resuspended in 10mL of complete medium. Each cell line was made up in  $5 \times 10^5$  cells/mL in 4 mL of complete medium. One well was set up as the blank that contained culture medium only. A standard growth curves was prepared by serial dilution of cells at:  $6.25 \times 10^3$ /mL,  $1.25 \times 10^4$ /mL,  $2.5 \times 10^4$ /mL,  $5 \times 10^4$ /mL,  $1 \times 10^5$ /mL, and  $2.5 \times 10^5$ /mL. 200  $\mu$ l of cell suspension from each dilution was transferred into a 96-wells plate in triplicate. After overnight incubation with 5% CO<sub>2</sub> at 37°C, the cells were stained with MTT assay as described in section 2.8.2. The cell proliferation assay was run for six days at one plate per day.



## 2.8.2 Assessing Cell Numbers Using MTT Assay

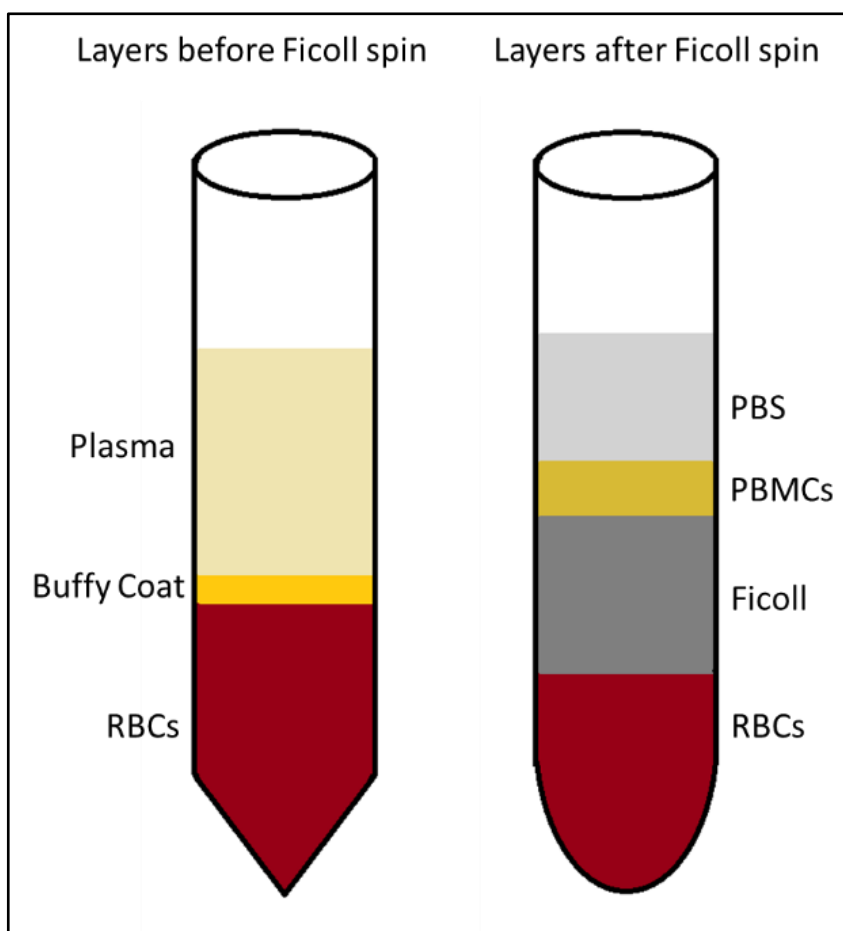
MTT stock solution was prepared at a concentration of 5 mg/mL and stored at 4°C. 50 µl of MTT stock solution, at each time point, was added to each well and the cells were incubated at 37°C, 5% CO<sub>2</sub> for 4 h. 200 µl of DMSO was added to each well, including the blank control wells after, which the medium was removed. The plate was incubated for 10 minutes at 37°C, in 5% CO<sub>2</sub> and the optical density read at 570 nm in a SPECTROstar Nano plate reader. The growth rate for each cell line was compared against the standard curve to obtain the number of cells/well each day for six days. Graphs of cell number/time were plotted.

## 2.9 Blood Sample Collection and Preparation

This study has been carried out under ethical approval from the England Health Research Authority National Research Ethics Service Committee, North West-Greater Manchester West [REC reference: 11/NW/0593] and East of England-Essex [REC reference 15/EE/0051] Ethics Committees. Informed consent was obtained for all participants prior to sample collection. Blood samples were drawn by phlebotomists in the blood collection area at the RLBUHT, following a standard safety protocol. Samples of blood (approximately 25mL) were obtained, using one 9mL S-Monovette® K3E, Potassium EDTA tubes and were inverted gently for 10 times, and kept in a standing position at room temperature. The date of collection and time were written on the participant's medical form and on the collected tubes. All samples were labeled with a unique identification number (ID) composed of six digits, made up from the year, month and the date of collection, followed by two letters, either OP for osteoporosis or HC for healthy volunteer, and two digits from the participant serial number.

## **2.10 Isolation of Peripheral Blood Mononuclear Cells [PBMCs]**

Plasma was isolated from the blood specimens by centrifuging the EDTA tube containing samples at 1000 g using Thermo Heraeus Multifuge 3SRT centrifuge at room temperature for 30 min. The upper layer containing the rest of the plasma, the buffy coat, and red blood cells were then transferred to 15 mL Tubes and diluted with the same volume of Dulbecco's Phosphate-Buffered Saline (DPBS). 3 mL of Ficoll-premium (1.077g/mL) solution was added into the bottom of a Greiner Bio-One 10 mL Leucosep® tube and centrifuged at room temperature for 1 min at 1000 g, (Figure 2-2). 6 mL of PBS-diluted blood samples were carefully added down the side of the Leucosep® tube held at 45 degrees and the tube was centrifuged at 200 g at RT for 30 min. The interface layer containing the PBMCs was then collected into a new sterile 15 mL Tubes, and washed with PBS at room temperature, followed by centrifuging at 200g at RT for 5 min and the supernatant was discarded. Cell pellets were then suspended with 10 mL of cold ammonium-chloride-potassium lysing buffer, and incubated at 4°C for 1 min, followed by centrifugation at 200 g for 5 min at 4°C. The pellets were washed twice using 10 mL PBS, and centrifuged at 1000g at room temperature for 5 min. Finally, the cell pellets were suspended in 0.5 mL PBS, and transferred into 1.5 mL Microcentrifuge tubes and centrifuged at 1000 g at 4°C for 2 min to form a tight cell pellet, which was stored at -80°C until further analysis.



**Figure 2-2: Isolation of PBMCs using ficoll density gradient.**

Tube was centrifuged to obtain the buffy coat which was then transferred to a new tube containing Ficoll. The remaining PBS was removed and PBMCs were collected in 1.5 mL Microcentrifuge tubes

## 2.11 Real-Time Quantitative PCR (RT-qPCR)

### 2.11.1 First-strand cDNA Synthesis

First-strand cDNA was synthesized from purified RNAs using the RT-PCR kit (Qiagen) according to the manufacturer's methodology. 500 ng of purified total RNA was mixed with 2  $\mu$ l Buffer GE, and RNase-free water added to a final volume of 10  $\mu$ l in a 0.5 mL tube (Table 2-6) and incubated in a Bio-Rad T100 thermal cycler at 42°C for 5 min, then placed immediately on ice for at least 1 min. 10  $\mu$ l of reverse-transcriptase mixture, as shown in Table 2-7, were added to each tube containing genomic DNA elimination mix and gently mixed by pipetting up and down. Reverse Transcriptase (RT) was replaced by RNase-free water for the no reverse transcription control (NRT) preparation. The RNA reverse transcription reaction mixtures, of final volumes of 20  $\mu$ L, were incubated at 42°C for exactly 45 min. and immediately the reaction was stopped by incubating at 95°C for 5 min, then centrifuged for 10 seconds to bring the contents to the bottom of the tubes. The resulting first-strand cDNA products were stored frozen at -20°C and samples were used as a template for RT-qPCR amplification.

**Table 2-6 Reaction mixture for genomic DNA elimination**

<b>Component</b>	<b>Amount</b>
Total RNA 500 ng	$\mu$ L Variable
Buffer GE	2 $\mu$ l
RNase-free water	Variable
<b>Total volume</b>	<b>10 <math>\mu</math>l</b>

**Table 2-7 Reaction mixture for preparing reverse-transcription**

Component	Volume for 1 reaction
RNase-free water	3 $\mu$ l
Control P2	1 $\mu$ l
RE3 Reverse Transcriptase Mix	2 $\mu$ l
5x Buffer BC3	4 $\mu$ l
<b>Total volume</b>	<b>10 <math>\mu</math>l</b>

First-strand cDNA for RT-qPCR of microRNAs was synthesized from purified RNAs using miScriptII RT-PCR kit (Qiagen).: 500 ng of total RNA was mixed with 4  $\mu$ l of 5x miScript HiSpec Buffer and 2  $\mu$ l of 10x miScript Nucleics Mix in a final volume of 20  $\mu$ l with Reverse Transcriptase (RT) as describe in Table 2-8. RT was replaced by RNase-free water for the no-RT control preparation. The reaction mixtures were incubated at 37°C for 60 min followed by incubation for 5 min at 95°C. The resulting reverse transcription products were stored frozen at -20°C and were used as a template for RT-qPCR amplification of microRNAs.

**Table 2-8: First-strand cDNA reaction mixture for preparing microRNA**

Component	Volume for 1 reaction
RNase-free water	$\mu$ L Variable
5x miScript HiSpec Buffer	4 $\mu$ L
10x miScript Nucleics Mix	2 $\mu$ L
500 ng of Total RNA	$\mu$ L Variable
miScript Reverse Transcriptase Mix	2 $\mu$ L
<b>Total Volume</b>	<b>20 <math>\mu</math>L</b>

### 2.11.2 Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR of mRNAs was performed by adding 1  $\mu$ l of diluted (1:20) RNA reverse transcription products to 1x RT<sup>2</sup> SYBR Green Mastermix, 0.4 $\mu$ M of RT<sup>2</sup> qPCR Primer in a final volume of 10  $\mu$ l (Table 2-9). The RT-qPCR mixtures were incubated in 96 well plates at. at 95°C for 10 min followed by 35 cycles of 2 steps cycling; annealing at

95°C for 15 second followed by an extension step for 15 seconds at 60°C. Melting curve analysis was performed (10 seconds at 95°C, 65°C for 60 seconds and 97°C for 1 seconds) to detect the presence of nonspecific products and the primer dimers. All RT-qPCR reactions were performed in duplicate and a No-Reverse Transcriptase (NRT) control was included. RT-qPCR amplification was performed using a Roche Lightcycler 96® real-time PCR system. All primers used for RT-qPCR in this study were from QIAGEN as shown in Table 2-3.

**Table 2-9: Reaction mixture for detection of mRNA**

Component	Volume for 1 reaction
RT <sup>2</sup> SYBR Green Mastermix	5 µl
Diluted cDNA synthesis reaction (1:20)	1 µl
RT <sup>2</sup> qPCR Primer Assay (10 uM stock)	0.4 µl
RNase-free water	3.6 µl
<b>Total</b>	<b>10 µl</b>

Detection of the expression of miRNAs was performed by RT-qPCR using miScript SYBER kit [Qiagen] according to manufacturer's recommendation. 1µl of diluted cDNA (1:20) was mixed with 5µl of 2x QuantiTect SYBR Green PCR Master Mix, 1µl of 10x miScript Universal Primer (reverse primer), 1 µl of 10x Primer Mix (forward miScript primer), with a final volume of 10 µl, (Table 2-10). The RT-qPCR mixture were incubated at 95°C for 15 min followed by 45 cycles of 3 steps cycling; denaturations at 94°C for 15s, annealing at 55°C for 30s and an extension at 70°C for 30s, melting step (95°C for 10s, 65°C for 60s and 97°C). All RT-q PCR reactions were performed in duplicate for the miRNA of interest and a No-Reverse Transcriptase (NRT) control was included. RT-qPCR amplification was performed using a Roche Lightcycler 96® real-time PCR system. All miRNAs used for RT-qPCR in this study were from QIAGEN as shown in Table 2-4.

**Table 2-10 Reaction mixture for detection of miRNA**

<b>Component</b>	<b>Volume for 1 reaction</b>
2x QuantiTect SYBR Green PCR Master Mix	5 $\mu$ l
10x miScript Universal Primer	1 $\mu$ l
10x miScript Primer Assay	1 $\mu$ l
RNase-free water	2 $\mu$ l
Template of diluted cDNA (1:20)	1 $\mu$ l
<b>Total Mix</b>	<b>9 <math>\mu</math>l</b>

### 2.11.3 Analysis of RT-qPCR Data

Relative quantities of mRNA/miRNA were calculated using the  $\Delta\Delta C_t$  methods after normalization to the controls including Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) for mRNA or Small Nucleolar RNA, C/D Box 96A (SNORD96A) for microRNAs. The expression levels of mRNAs or MiRNA between the osteopaenia, osteoporosis and non-osteoporosis control were calculated using the  $\Delta C_t$  equation as well as the fold change ( $2^{-\Delta C_t}$ ) for each mRNA/miRNA (Bustin et al., 2005). Since  $C_t$  values greater than 35 were considered to be below the detection level of the reaction, (indicated as N/A, not detected), all mRNA/miRNA RT-qPCR reactions with  $C_t > 35$  were included in the analysis as undetected and assigned the  $C_t$  values of 35.

$$\Delta C_t = C_t \text{ Treated} - C_t \text{ control}$$

$$\Delta C_t = C_t \text{ Untreated} - C_t \text{ control}$$

$$\Delta\Delta C_t = \Delta C_t \text{ Treated} - \Delta C_t \text{ Untreated}$$

$$\text{Fold change} = 2^{-(\Delta\Delta C_t)}$$

## 2.12 Western Blotting

### 2.12.1 Preparation of Cell Lysates

After washed twice with PBS,  $5 \times 10^6$  cells were lysed with 1 mL of cold RIPA buffer (ThermoFisher, UK) and incubated on ice for 5 min with slight swirling of the plate occasionally. The lysate was then gathered to one side, using a cell scraper to be collected in 1.5 mL microcentrifuge tube before centrifuging at 100 g for 15 min to pellet the cell debris. The supernatant was resuspended in an equal volume of 2x Laemmli lysis buffer and then transferred to a new microcentrifuge tube. Protein samples were heated for 5 min at 95°C, chilled on ice for 2 min and centrifuged before loading onto an SDS polyacrylamide gel or stored at -20°C until further analysis.

### 2.12.2 Quantification of Total Protein in Cell Lysates

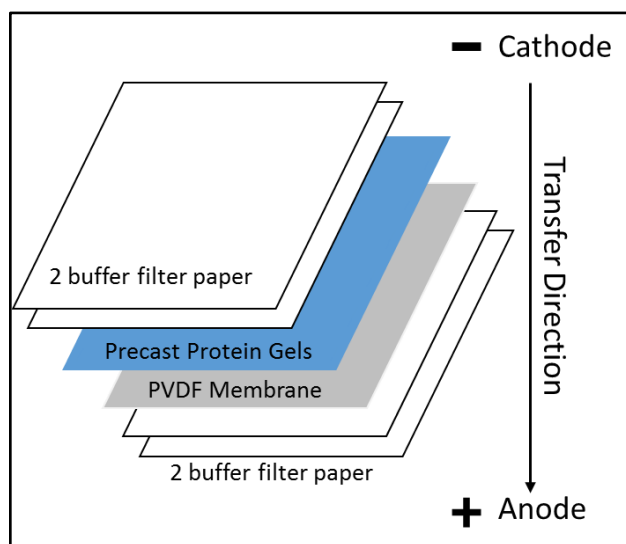
The total protein content of cell lysate was determined using the Qubit® Protein Assay Kit (Invitrogen Ltd, Paisely, UK) according to manufacturer's recommendation. Briefly, a stock Qubit™ working solution containing Qubit dye was prepared by diluting the Qubit™ protein reagent 1:200 in the dilution buffer. 10 µl of standard sample was added to 190 µl of working solution, while 1 µl of each experimental sample was added to 199 µl of working solution. All tubes were vortexed for 2-3 seconds and incubated at room temperature for 15 min. The fluorescence signal was measured on the Qubit® Fluorometer 2.0.

The protein concentration was calculated based on the amount of fluorescence signal directly proportional to the concentration of protein in the solution using a calibration of protein standards of known concentration.



### 2.12.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on 12% Mini-PROTEAN® TGX™ Precast Protein Gels using the Bio-Rad mini protean gel system. 20 µg of protein were loaded per lane of the gel alongside a lane containing protein molecular weight markers (Precision Plus Protein™ Dual Color Standard, BioRad). Gels electrophoresis was carried out at 200V and 30 mA for 40 min. After gel electrophoresis, proteins were electroblotted onto a PVDF membrane which had been activated by soaking in 100% methanol then equilibrated in transfer buffer. The blotting unit was assembled by placing components as shown in Figure 2-3. It was necessary to roll out the components to ensure there were no air bubbles between layers within the cassettes. Once the stacks were positioned on the cassette base, transfer was carried out at 1.5mA, 25 V, for 7 min.



**Figure 2-3: Schematic diagram illustrating the order to transfer protein from gel to PVDF membrane using Trans-Blot® Turbo™.**

The order started from bottom by 2 Filter papers then the PVDF membrane then the gel and lastly 2 filter papers)

After electroblotting, the PVDF membrane was shaken continuously in 5% (w/v) non-fat milk in Tris-Buffer Saline Tween-20 (TBST) for 1 hour at room temperature to block non-specific binding sites on the membrane. The membrane was then incubated with primary antibody in an appropriate concentration diluted in 5% non-fat milk in TBST overnight at 4°C under continuous shaking (list of primary antibodies, Table 2-1). The membranes were then washed three times in TBST for 10 mins each time with gentle rocking to remove unbound primary antibodies. The membrane was then incubated for one hour at room temperature with gentle shaking with the appropriate secondary antibody in an appropriate concentration, (Table 2-2), made up in 5% non-fat milk /TBST. The membrane was again washed three times in TBST for 10 min each time.

The levels of protein were detected using an enhanced Chemiluminescent Substrate kit (SuperSignal™ West Femto Chemiluminescent Substrate, Fisher Scientific) which measures the horseradish peroxidase (HRP) activity from the secondary antibodies. The ECL substrate kit contains ECL reagents 1 and 2 which were mixed in a 1:1 solution and applied to the membrane for 2-3 mins. The membrane was enclosed in a clear plastic sheet protector to prevent drying and signals were measured using BioRad ChemiDOCTM digital imager (BioRad, UK). Densitometric values of band intensity were taken using the BioRad Image Lab program.  $\beta$ -Actin was used as control to evaluate equal loading of samples.

## 2.13 Statistical Analyses

A variety of statistical tool were used to measure the numerical analytical data including IBM SPSS Statistic 22.0 (UK Head Office IBM United Kingdom Limited PO

Box 41, North Harbour Portsmouth Hampshire, PO6 3AU) and GraphPad Prism version 7 (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA).

Student's t-test was used to compare any differences observed between each experimental group and the control group. One-way ANOVA with 'Bonferroni's multiple comparisons test' was used to compare the mean between three or more groups. Two-way ANOVA 'Dunnett's multiple comparisons test' was used to compare the mean between different groups when different treatments were compared with the control.

SPSS Ver. 22.0 was used to calculate the area under the receiver operating characteristic (ROC) curve (AUC) diagnostic value of mRNAs and miRNAs of clinical samples. The cut-off points with the highest sensitivity and specificity were determined. Statistical significance was set at a p-value less than 0.05. In Figures P values were indicated by \*: (\*) <0.05, (\*\*) <0.01, (\*\*\*) <0.001, (\*\*\*\*), <0.0001(\*\*\*\*\*).

# **Chapter 3: Identification of Differentially- Expressed Hormone-Responsive Genes in Cultured Osteosarcoma Cells by RNA Sequencing**

### 3.1 Introduction

Osteoporosis is a common bone disease among the elderly that occurs when the body loses bone mass, which results in bone weakness and fracture. Until a fracture occurs there are no symptoms (Rachner et al., 2011). In postmenopausal women, osteoporosis is mainly caused by a deficiency of estrogen resulting in a high risk of suffering from the disease (Nih Consensus Development Panel on Osteoporosis Prevention and Therapy, 2001). Estrogen plays a significant role in the growth and maturation of bone and in the regulation of bone remodelling (Hofbauer et al., 1999). The major physiological effect of estrogen is to inhibit bone resorption by osteoclasts and induce bone formation by osteoblasts (Hofbauer et al., 1999) (Riggs, 2000). The effect of estrogen, on bone and other target tissues, is mediated by estrogen receptors  $\alpha$  and/or  $\beta$  (ER $\alpha$ , ER $\beta$ ), and these receptors are encoded by different genes, located on human chromosomes 6 and 14, respectively (Fitzpatrick, 2006). Once estrogen bind to these receptors in the nucleus, gene expression is modulated, either by the complex binding directly to specific response elements, or indirectly by protein-protein interaction in the regulatory regions of the estrogen-target genes (Tsai and O'Malley, 1994). However, the influence of these receptors on the pattern of gene expression in the bones of postmenopausal women, which might be associated with the development of osteoporosis, is still unknown (Chen et al., 2004).

Due to the difficulty of isolating, in an intact state, the cellular components of bone (Damien et al., 1998, Aguirre et al., 2007), cultured osteosarcoma cells can be used as a bone related system (Denger et al., 2008, Pacheco-Pantoja et al., 2011). The *in vitro* osteosarcoma cells used in this study, (Mg-63, TE-85, and Saos-2), represent different

stages of osteoblastic development, where Mg-63 is identified as the cell line being the least mature, Saos-2 as the most differentiated and TE-85 may have a level of maturity somewhere between Mg-63 and Saos-2 (Pacheco-Pantoja et al., 2011).

### **3.1.1 Aim**

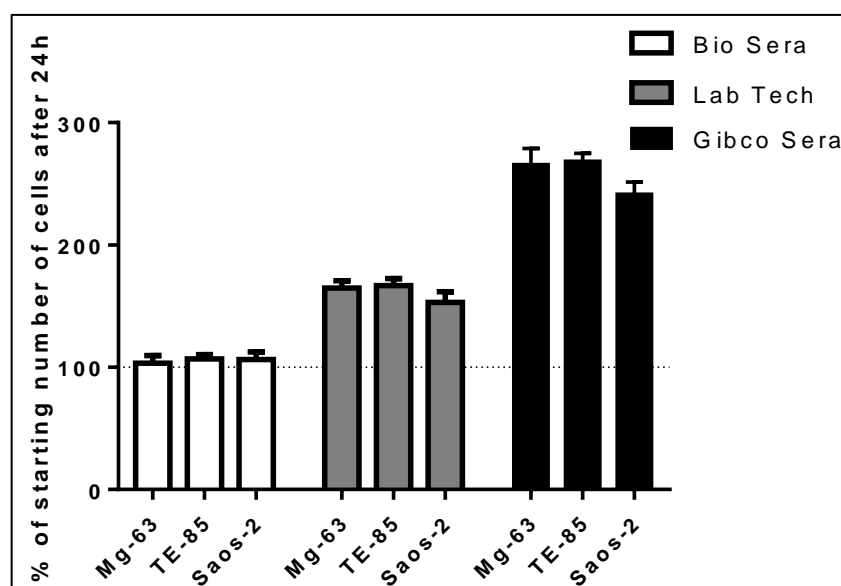
The purpose of this chapter is to identify differentially-expressed estrogen-responsive genes using cultured osteosarcoma cells by RNA sequencing. This aim will be achieved by performing the following experiments:

- Establishing an estradiol ( $\beta$ -estradiol) responsive model of cultured osteosarcoma cells (Mg-63, TE-85, and Saos-2).
- Investigating the effect of  $\beta$ -estradiol on the proliferation of the cultured osteosarcoma cells.
- Identifying, using RNA-sequencing, mRNAs of genes that are differentially-expressed when the osteosarcoma cells are treated with estradiol.

## 3.2 Result

### 3.2.1 Establish a Hormone-Responsive Model in Cultured Osteosarcoma Cells

Different batches of foetal bovine serum (FBS) from different companies were tested to identify the serum batch that is suitable to support the growth of cultured osteosarcoma cells. The results showed that 10 % Bio Sera FBS did not increase the number of cells at all over 24 h. 10 % of LabTech serum in the medium showed an increase in cell number by only 60 %. However, 10 % of Gibco FBS in the culture medium increased the accumulation of all three cultured cells by more than two-fold over 24 h, compared to the starting number of cells (Figure 3-1). Therefore, 10 % of Gibco FBS was used for the rest of the project.



**Figure 3-1: Change in cell numbers of osteosarcoma cell lines tested with different batches of foetal bovine serum.**

Three osteosarcoma cell lines were cultured and the number of cells was determined after 24h of culture and compared with the number of cells at the start, which was set at 100. Each bar represents the mean  $\pm$ SD of triplicate experiments.

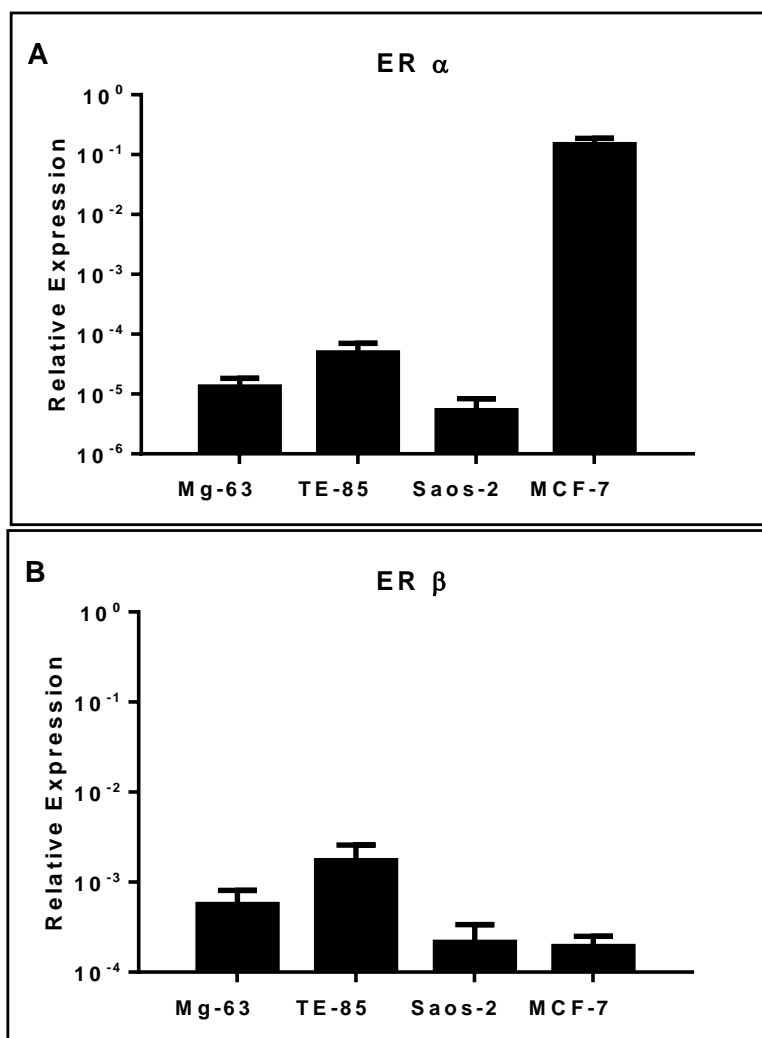
### **3.2.2 Estrogen Receptors $\alpha$ and $\beta$ in the Cultured Osteosarcoma Cells**

The purpose of this part of this study was to detect the presence of ER  $\alpha$  and  $\beta$  mRNAs/proteins in the Mg-63, TE-85, and Saos-2 osteosarcoma cell lines, as well as in MCF-7 cells, a positive control for ER $\alpha$ , using real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot.

#### **3.2.2.1 The Levels of Estrogen Receptors $\alpha$ and $\beta$ mRNAs in the Cultured Osteosarcoma Cells**

Total RNAs were isolated from the Mg-63, TE-85 and Saos-2 osteosarcoma cell lines and the MCF-7 cells and treated with DNase to remove traces of genomic DNA contamination before reverse transcription. Levels of ER  $\alpha$  and  $\beta$  mRNAs were determined by RT-qPCR using specifically designed primers (Table 2-3). The levels of mRNA in cultured osteosarcoma cell lines were compared to the positive control, MCF-7 cells, using the  $2^{-\Delta C_t}$  method with GAPDH as a housekeeping control gene. The results showed that the ER  $\alpha$  mRNA was detected in Mg-63, TE-85 and Saos-2 cells (Figure 3-2A), but at less abundant levels than in the positive control cells, MCF-7. However, in contrast, the levels of ER  $\beta$  mRNA were much higher in all three osteosarcoma cell lines than that in the MCF-7 cells, (Figure 3-2B). The results show that both ER  $\alpha$  and  $\beta$  mRNAs are present in the cultured osteosarcoma cells, with ER  $\beta$  being more abundant than ER  $\alpha$ .



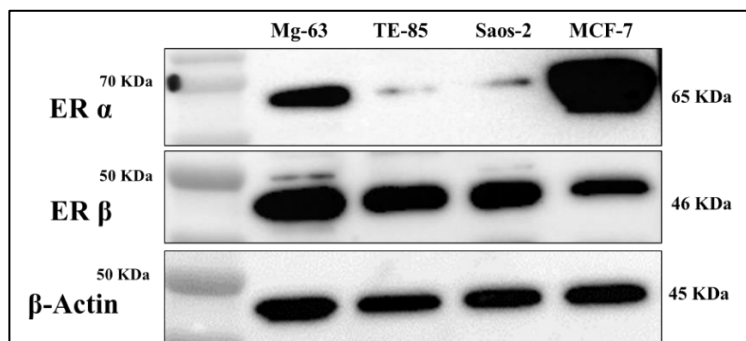


**Figure 3-2: The relative expression levels of estrogen receptor  $\alpha$  and  $\beta$  mRNAs in osteosarcoma cell lines and control MCF-7 cells** mRNAs for ER $\alpha$  (A) and ER $\beta$  (B) were detected using RT-qPCR and relative expression levels of mRNA compared to MCF-7 cells were determined using the  $2^{-\Delta C_t}$  method with GAPDH as a constitutively-expressed control mRNA. Each bar represents the mean  $\pm$ SD (n=3). All RT-qPCR data were logarithmically transformed.

### 3.2.2.2 Estrogen Receptor $\alpha$ and $\beta$ Proteins in the Cultured Osteosarcoma Cells

The presence of estrogen receptor  $\alpha$  and  $\beta$  proteins in osteosarcoma cell lines was determined by western blot, using antibodies for estrogen receptors  $\alpha$  or  $\beta$ . A  $\beta$ -actin antibody was used as a housekeeping control to standardise between samples, (Table 2-1 and Table 2-2).

Immunoblotting for ER  $\alpha$  and  $\beta$  protein in the osteosarcoma cell lines, Mg-63, TE-85 and Saos-2, and control MCF-7 cells, revealed distinct bands at 65 and 46 kDa corresponding to the sizes of the ER  $\alpha$  and  $\beta$  proteins, respectively (Figure 3-3) (Wang et al., 2014, Park et al., 2017). The results showed that, as with the mRNA measurements by RT-qPCR, the levels of ER  $\alpha$  protein in the osteosarcoma cells were not as abundance as in the positive control cells, MCF-7. The ER  $\alpha$  protein is present in the cultured osteosarcoma cell line Mg-63 cells, but the levels of ER  $\alpha$  protein were much lower in both TE-85 and Saos-2 cells compared to Mg-63 cells. The ER  $\beta$  protein was detected in all three cultured osteosarcoma cell lines, Mg-63, TE-85 and Saos-2 cells, and showed similar/greater levels as those in MCF-7 cells (Figure 3-3).



**Figure 3-3 Western blot analysis of estrogen receptor  $\alpha$  and  $\beta$  in osteosarcoma and MCF-7 cell lines.**

ER  $\alpha$  and  $\beta$  protein bands at 65 and 46 kDa, respectively, were detected in osteosarcoma and MCF-7 cell lines.

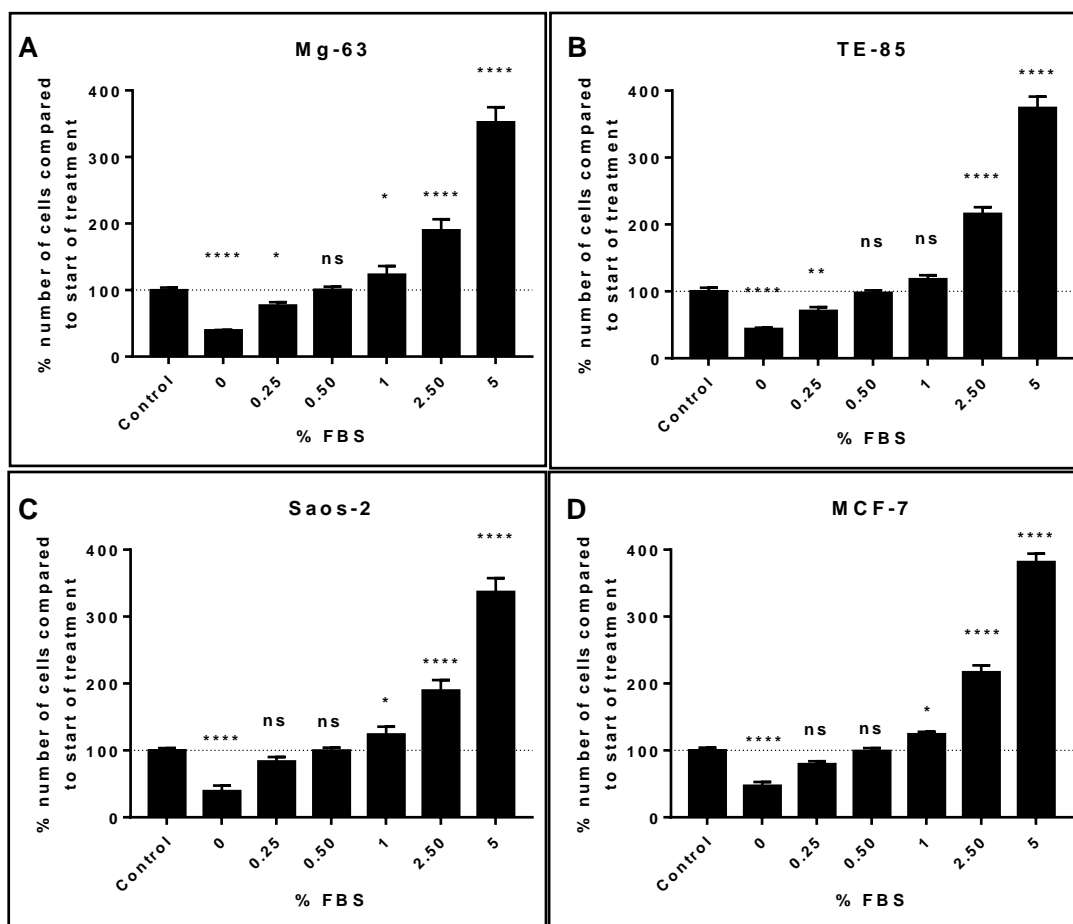
### **3.2.3 The Effect of Estradiol on the Proliferation of the Cultured Osteosarcoma Cells**

The effect of Estradiol on the proliferation of osteosarcoma cell lines and MCF-7 cells was evaluated using phenol red-free medium (RPMI) containing steroid-free FBS (Materials and Methods, Section 0).

#### **3.2.3.1 Optimization of the Rate of Growth on Osteosarcoma Cells Using In-house Hormone-Stripped FBS.**

The purpose of this optimization is to find the minimum concentration of hormone-stripped FBS that allows cell survival for a period of 6 days in the absence of steroid hormones, to ensure that cells can survive over this period without growing.

It was found that in 0.5 % hormone-stripped FBS, none of the cells showed any significant change in number compared to the starting number (all  $p > 0.99$ ) Thus, the cells stayed alive for 6 days without cell proliferation or cell death, whereas higher and lower concentrations of hormone-stripped FBS generally resulted in an increase or reduction in cell number relative to the starting number (Figure 3-4 and Table 3-1). The result demonstrates that RPMI supplemented with 0.5 % hormone-stripped FBS kept cells in a life state without any growth in Mg-63, TE-85, Saos-2 and MCF-7 cells compared to the starting number. Thus, 0.5% hormone stripped FBS was used in subsequent experiments.



**Figure 3-4: The effect of hormone-stripped medium on the growth of cell lines**

$1 \times 10^4$  cells/  $\text{cm}^2$  were grown initially to 60 % confluence in complete medium, washed twice with PBS and then transferred into RPMI containing different concentrations of hormone-stripped FBS to deplete the endogenous steroids for 6 days. Medium was changed daily. The number of cells was calculated by counting cells after 6 days (A. Mg-63, B. TE-85, C. Saos-2 and D. MCF-7). Each bar represents the mean percentage change  $\pm$ SD (n=3). The control is the mean number of cells before treatment with stripped FBS, which was normalised to 100%. ns=non-significant, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ .

**Table 3-1: The effect of hormone-stripped FBS on the growth of cell lines**

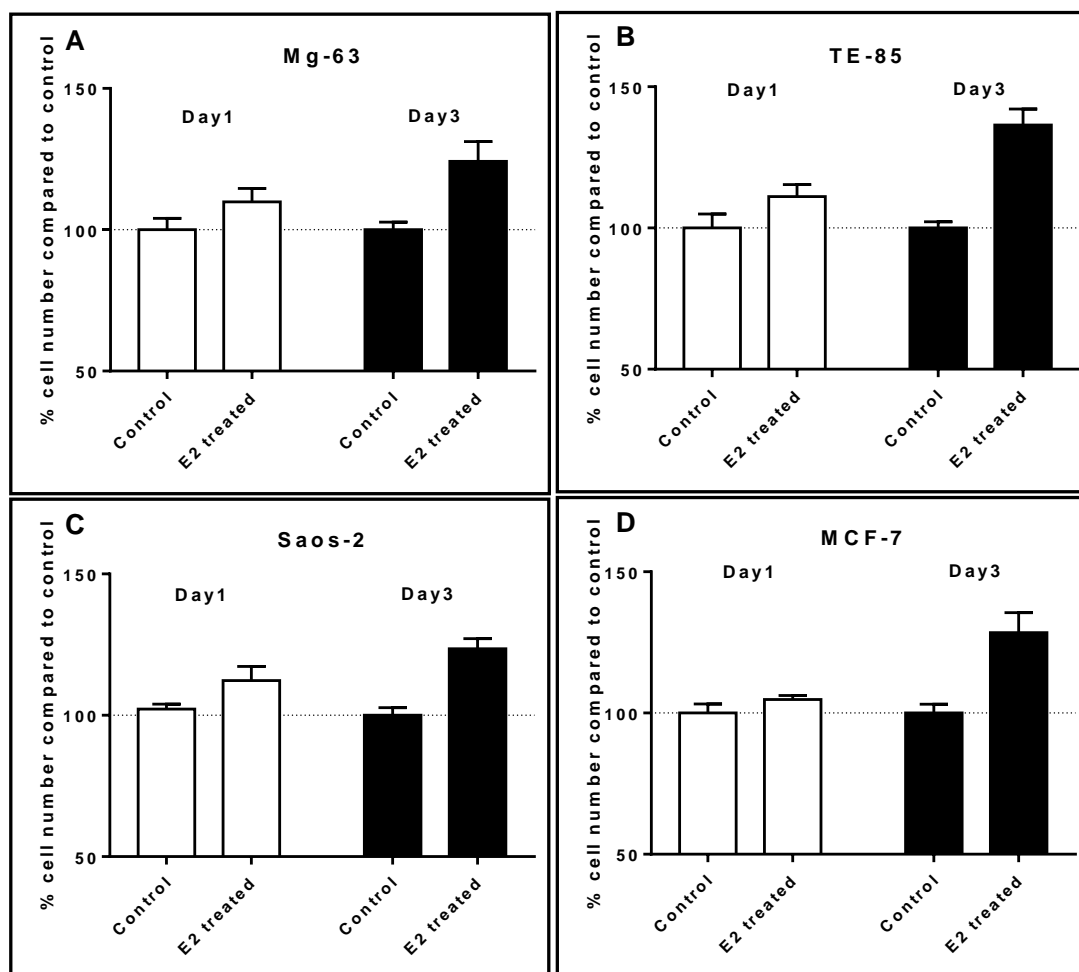
<b>Cells</b>	<b>Hormone-stripped FBS concentrations</b>	<b>Rate of growth (Mean Difference to the Control %)</b>	<b>P Value</b>
<b>Mg-63</b>	0.00% vs. Control	-60.46	<0.0001
	0.25% vs. Control	-23.09	0.048
	0.50% vs. Control	<1	>0.99
	1% vs. Control	23.15	0.0474
	2.50% vs. Control	89.89	<0.0001
	5% vs. Control	252.1	<0.0001
<b>TE-85</b>	0.00% vs. Control	-56.17	<0.0001
	0.25% vs. Control	-29.19	0.006
	0.50% vs. Control	-2.16	>0.99
	1% vs. Control	18.15	0.2102
	2.50% vs. Control	115.8	<0.0001
	5% vs. Control	274.1	<0.0001
<b>Saos-2</b>	0.00% vs. Control	-60.85	<0.0001
	0.25% vs. Control	-16.66	0.3134
	0.50% vs. Control	<1	>0.99
	1% vs. Control	23.69	0.0398
	2.50% vs. Control	89.44	<0.0001
	5% vs. Control	236.8	<0.0001
<b>MCF-7</b>	0.00% vs. Control	-52.86	<0.0001
	0.25% vs. Control	-20.35	0.1122
	0.50% vs. Control	<1	>0.99
	1% vs. Control	24.33	0.0323
	2.50% vs. Control	117	<0.0001
	5% vs. Control	284.9	<0.0001

The control is the mean number of cells for each cell line before treatment with stripped FBS which was normalised to 100%. Two-way ANOVA, (Dunnett's multiple comparisons test) was used to calculate the rate of growth compared to the control

### 3.2.3.2 The Effect of Estradiol on the Proliferation of Osteosarcoma Cells

The effectiveness of the step-down period prior to adding  $\beta$ -estradiol was optimised by incubating cells at a density of  $1 \times 10^4$  cells/  $\text{cm}^2$  for 1 or 3 days in phenol-free RPMI medium containing 0.5% hormone-stripped FBS, followed by the addition of 1 nM of  $\beta$ -estradiol (Dohi et al., 2008) into the step-down medium and incubation for 48hr (Materials and Methods, Section 0). Step-down medium without  $\beta$ -estradiol was used for control cells. The results (Figure 3-5 and Table 3-2) showed that incubating cells for 1 day in the step-down medium, followed by 48 h in the presence of  $\beta$ -estradiol, was insufficient for estrogen to stimulate cell numbers of Mg-63 and MCF-7 cells compared to untreated controls ( $p = 0.07$  and  $0.4$  respectively). In contrast, TE-85 and Saos-2 cells showed a significant increase in cell number compared to the control untreated cells ( $p = 0.03$  and  $0.0016$ ) On the other hand, following step-down, incubation of the cells for 3 days with 1 nM  $\beta$ -estradiol significantly increased cell numbers of all four cell lines Mg-63, TE-85, Saos-2 and MCF-7 ( $p = 0.006$ ,  $<0.0001$ ,  $0.0002$  and  $<0.0001$  respectively).

In order to produce more cells for later RNA isolation, larger dishes with  $25 \text{ cm}^2$  growth area were used. Cells were seeded ( $3 \times 10^4$  cells/  $\text{cm}^2$ ) equally to each flask and the same protocol was followed as with the  $9.5 \text{ cm}^2$  dishes. There was a significant increase in cell numbers for all 4 cell lines after treatment with  $\beta$ -estradiol for 48 h compared with untreated cells ( Figure 3-6; Mg-63,  $p < 0.0001$ ; TE-85,  $p = 0.0034$ ; Saos-2,  $p = 0.0003$ ; MCF-7,  $p < 0.0001$ ). Therefore, the results obtained with the larger dishes were consistent with those obtained with the pilot 6-well plates.



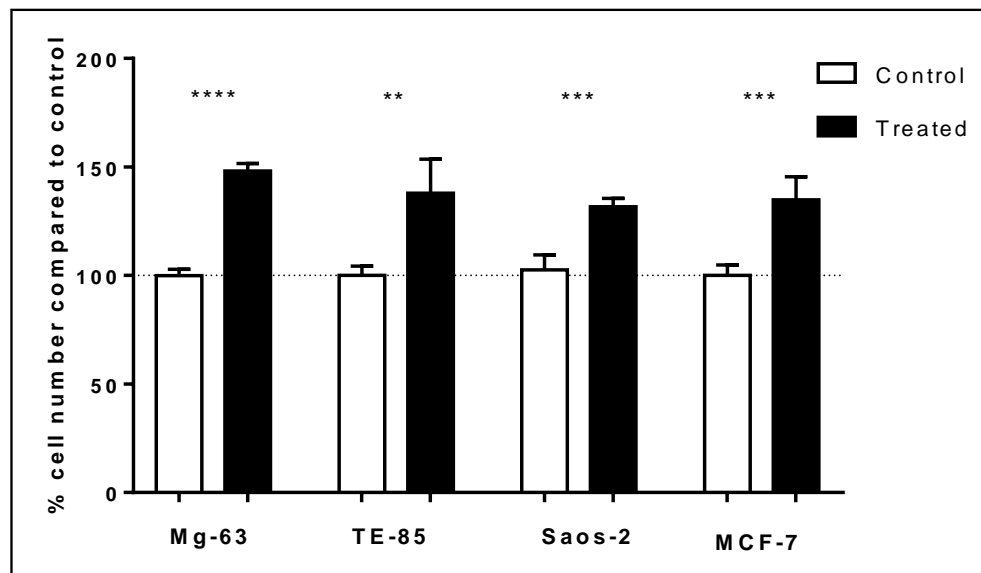
**Figure 3-5: The effect of 1 nM  $\beta$ -estradiol on the proliferation of cell lines.**

The indicated cell lines were incubated in step-down medium for 1 or 3 days before treatment with 1 nM  $\beta$ -estradiol for 48 h. Control cells were incubated for 48 h without  $\beta$ -estradiol normalised to 100%. White bars, cells incubated for 1 day; Black bars, cells incubated for 3 days. Each bar represents the mean  $\pm$ SD (n=3). \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

**Table 3-2:  $\beta$ -estradiol on Proliferation of Osteosarcoma Cells:**

Cells	Incubation Time	Rate of growth relative to the Control (%)	P Value
Mg-63	1 Day	110	0.0737
	3 Days	124	0.0006
TE-85	1 Day	111	0.0306
	3 Days	136	<0.0001
Saos-2	1 Day	117	0.0016
	3 Days	124	0.0002
MCF-7	1 Day	105	0.408
	3 Days	129	<0.0001

1 nM  $\beta$ -Estradiol induced the proliferation of cells incubated in **0.5%** hormone-stripped medium for 1 or 3 days using 6 well-plates.

**Figure 3-6: The effect of 1 nM  $\beta$ -estradiol on the proliferation of cell lines scaled up in TC-25 dishes.**

The indicated cell lines were incubated in  $\beta$ -estradiol stripped medium in TC-25 flasks for 3 days before adding  $\beta$ -estradiol. Control cells were incubated for 48 without  $\beta$ -estradiol, which was set at 100. Live cell numbers were counted after 48 h. Each bar represents the mean  $\pm$ SD (n=3)\*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001.



### **3.2.4 Identify Differentially Expressed Estrogen-Responsive Genes in Cultured Osteosarcoma Cells by RNA-Sequencing**

#### **3.2.4.1 RNA Purity and Quality Control**

Total RNAs were extracted from cells lines by using a combination of TRIzol® reagent and PureLink® RNA Mini Kit (Ambion, UK) (Materials and Methods, Section 2.3.1) and were checked for purity and yield using a NanoDrop™ 2000 spectrophotometer (Materials and Methods, Section 2.3.3). All isolated RNAs exhibited 260/280 and 260/230 ratios that exceeded the benchmark of 2 (Evans, Chomczynski and Sacchi, 2006) (Table 3-3), a requirement for successful RNA sequencing.

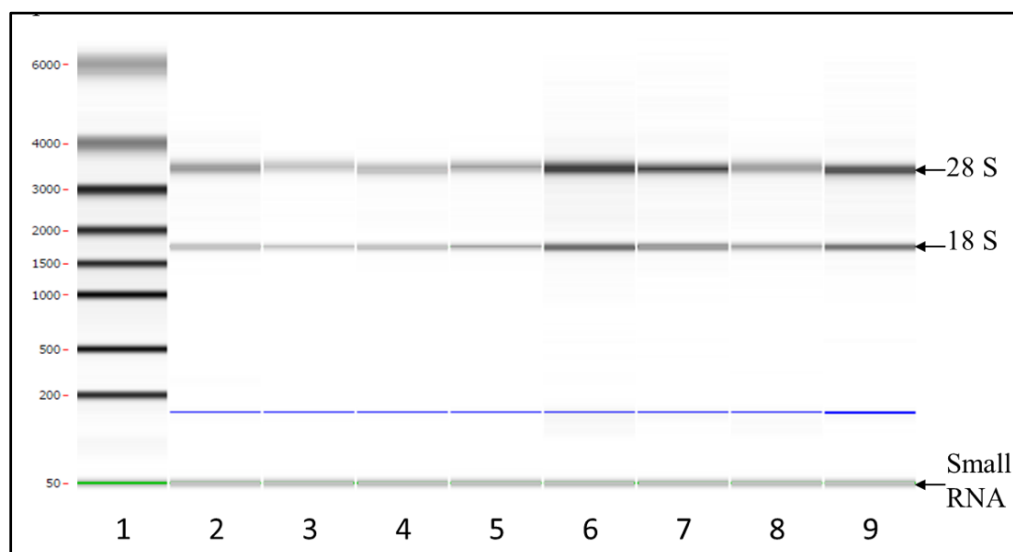
In order to identify differentially-expressed mRNA between control (untreated) and  $\beta$ -estradiol-treated osteosarcoma and MCF-7 cells, 6  $\mu$ g of the total RNA for each cell line was subjected to RNA sequencing by The Genome Analysis Centre (TGAC) (Norwich, UK). Additional RNA quality assessment was performed using an RNA6000 Nano LabChip on an Agilent 2100 bioanalyzer by TGAC (Table 3-3, Figure 3-7 and Figure 3-8). The data showed that all the isolated RNAs from all cell lines exceeded the benchmark ratio of 28S to 18S ribosomal RNA ratio of  $>2$  (Table 3-3) (Skrypina et al., 2003). The fluorescence plots with two peaks representing 18S and 28S ribosomal RNAs of control and treated cell lines are shown in Figure 3-8. The RNA integrity number (RIN), which was calculated by TGAC for each isolated RNA, showed at least 9.4 out of 10. The conclusion is that the total RNA was intact and no degradation was

observed (Schroeder et al., 2006) and these high quality RNAs purified from all the experimental treated or control cultured cells were used for RNAs sequencing.

**Table 3-3: Quality check of RNA isolated from control and treated cell lines**

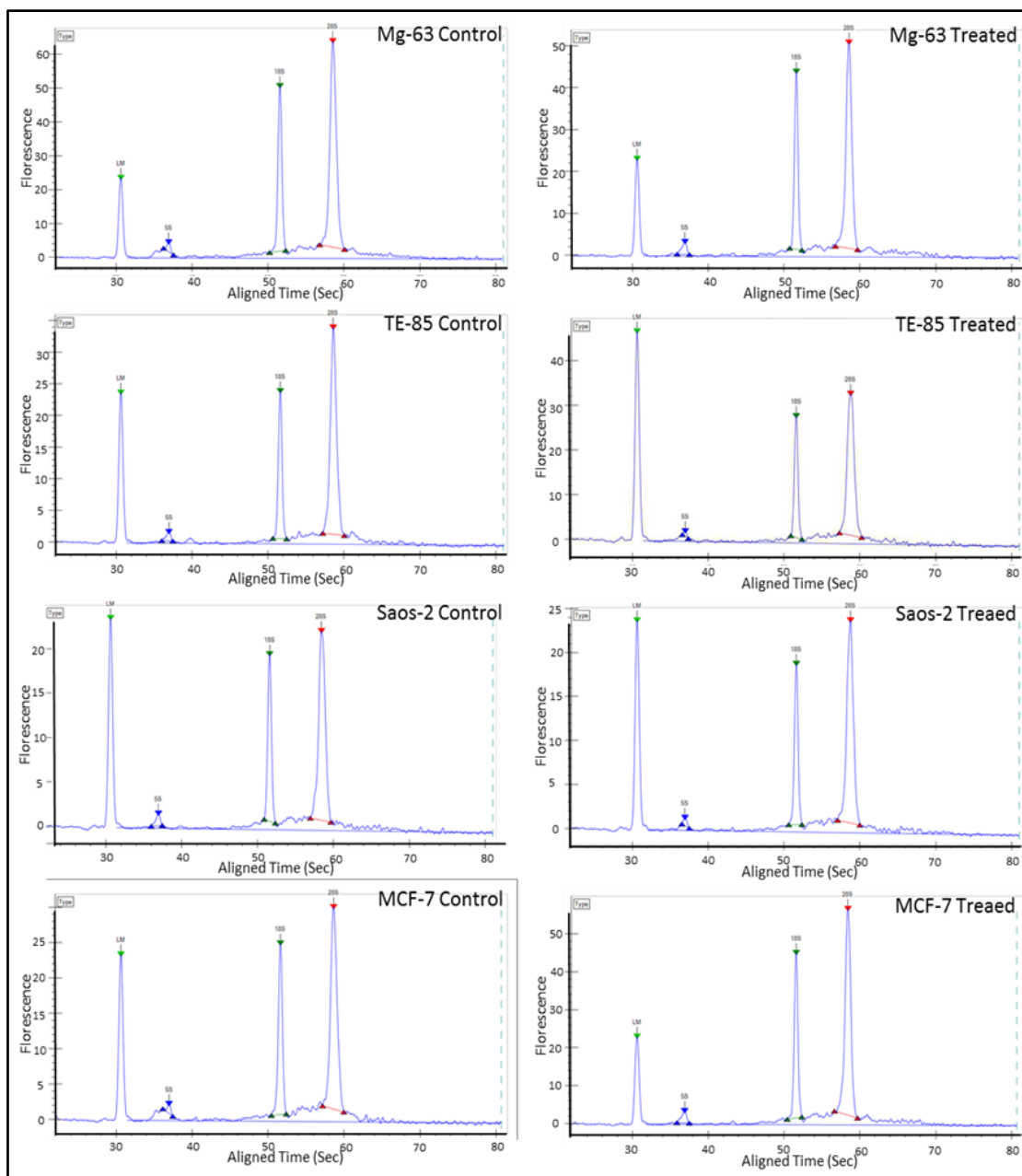
Sample ID	A260/280	A260/230	RIN	rRNA Ratio [28S/18S]
TE-85-Control	2.06	2.04	9.9	2.33
TE-85-Treated	2.02	2.02	9.6	2.28
Saos-2-Control	2.05	2.07	9.5	2.05
Saos-2-Treated	2.04	2.03	9.7	2.2
Mg-63-Control	2.1	2.12	9.4	2.05
Mg-63-Treated	2.1	2.07	9.5	1.93
MCF-7-Control	2.11	2.22	9.5	2.0
MCF-7-Treated	2.09	2.25	9.7	2.06
<b>Benchmark value</b>	2 (Evans)	2 (Evans)	10 (Schroeder et al., 2006)	2 (Skrypina et al., 2003)

The quality check of RNA was performed using a NanoDrop™ 2000 spectrophotometer and an Agilent 2100 Bioanalyzer. The RIN and rRNA ratios were determined by TGAC



**Figure 3-7: Quality of RNA isolated from both control and treated cells using Agilent 2100 Bioanalyzer.**

The gel electropherogram images for Ladder (Lane 1), TE-85 Control (Lane 2), TE-85 Treated (Lane 3), Saos-2 Control (Lane 4), Saos-2 Treated (Lane 5), Mg-63 Control (Lane 6), Mg-63 Treated (Lane 7), MCF-7 Control (Lane 8) and MCF-7 Treated (Lane 9). Data generated by TGAC.



**Figure 3-8: Quality check of total RNA samples by the Agilent 2100 bioanalyzer.**

Horizontal axis (Monroe et al.) denotes the aligned time in seconds, and the vertical axis (FU) denotes the fluorescent units (measure of the intensity). All panels showed two peaks of 18S and 28S ribosomal RNAs of each control sample, whereas right plots with two peaks of 18S and 28S ribosomal RNAs of each treated sample. Data generated by TGAC.

### 3.2.4.2 RNA sequencing Quality Control (QC)

Following cDNA synthesis, 8 Illumina TruSeq RNA libraries, both control and treated for each of the 4 cell lines, were generated by TGAC and sequenced across two lanes of a HiSeq2000 with 100 bp paired-end reads. Reads were mapped to the reference human genome, using Tophat software version 2.0.12.

An initial step in the analysis of RNA-Seq data evaluated the quality of sequencing, trimming and mapping, using the MultiQC tool of RNA-seq QC software. The mapping of the sequencing data is a useful quality control step to evaluate the quality of the individual RNA libraries. Alignment of RNA-Sequencing reads to transcriptome reference [Human genome build 38] was carried out as shown in Table 3-4. The table summarizes the mapping result by showing the total number of reads obtained and percentage of reads that mapped to the human reference genome. On average reads, across all samples,  $52.3 \pm 8$  million reads were obtained for each sample, with  $88.19 \pm 2$  % identical to the human reference genome. Hence, all samples achieved the 70-90 % benchmark (Conesa et al., 2016) for sequences mapping onto the reference human genome.

**Table 3-4: Summary of the mapping results to the reference human genome build 38 for each library.**

<b>Sample ID</b>	<b>Total read count</b>	<b>Mapping percentage to the Reference</b>
<b>TE-85-Control</b>	52140759	89 %
<b>TE-85- Treated</b>	59171549	89.6 %
<b>Saos-2- Control</b>	54013583	89.3 %
<b>Saos-2- Treated</b>	46404274	89.4 %
<b>Mg-63- Control</b>	54445889	88.7 %
<b>Mg-63- Treated</b>	34771768	83.7 %
<b>MCF-7- Control</b>	57943863	88.3 %
<b>MCF-7-Treated</b>	59516355	87.5 %

### 3.2.4.3 Analysis of RNA Sequencing

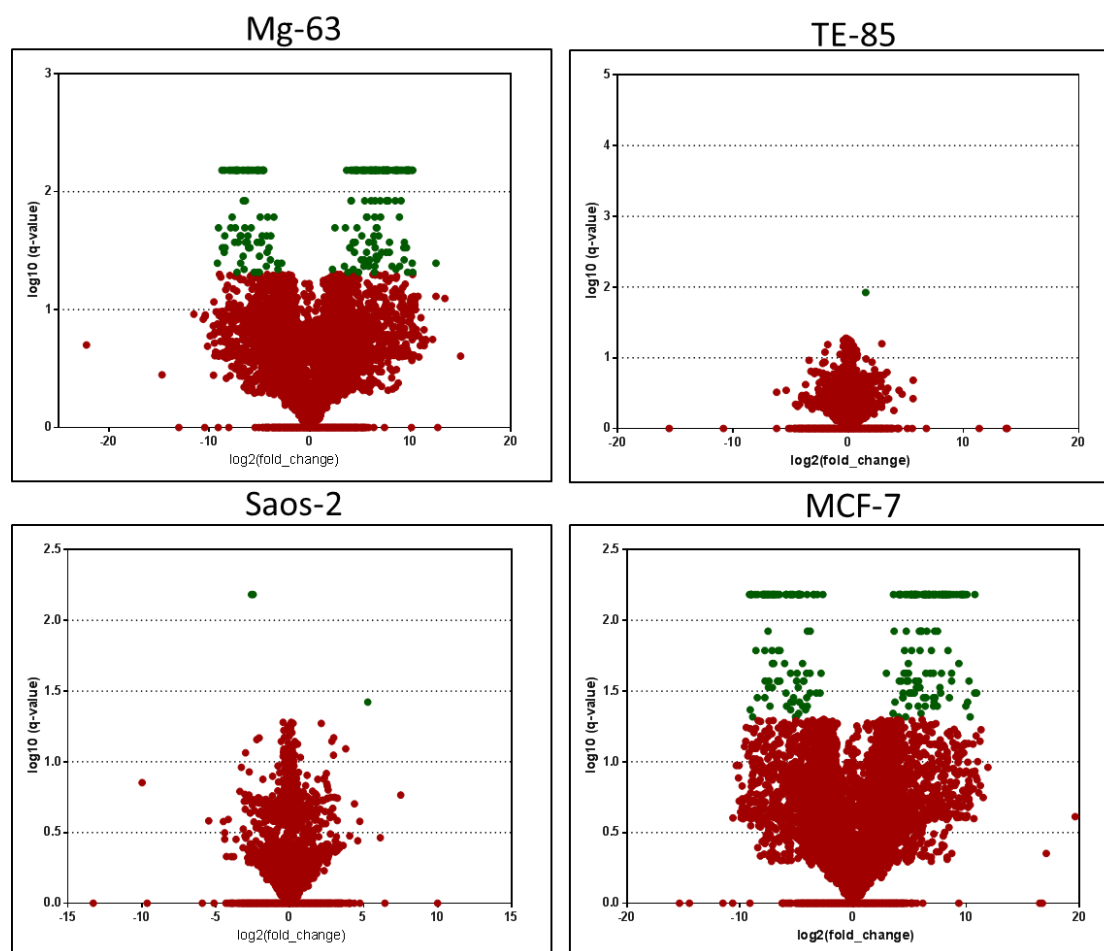
#### 3.2.4.3.1 Bioinformatics Analysis of RNA-Seq

Bioinformatics analysis of the RNA-seq data was performed by TGAC (Norwich, UK) using Cufflinks software to identify differentially-expressed genes between control and  $\beta$ -estradiol -treated cells. The list of differentially-expressed genes was narrowed to those significantly changed with q value  $< 0.05$  and  $\pm 2$  fold changed. The q value is an adjusted p-value using Benjamini-Hochberg False Discovery Rate (FDR) approach to correct for multiple testing.

Volcano plots were constructed to provide a quick visual representation of the result from RNA Seq (Figure 3-9) for each cell line. There are 2 regions of interest in the Volcano plot. Those points that are found near the top of the plot have high statistical significance, with q value of  $< 0.05$ . Points shown as green dots represent genes that are significantly changed by  $\pm 2$  fold. However, dots in red represent genes that are not significantly changed with q value of  $> 0.05$ .

RNA-Seq identified 154 genes that were up-regulated by at least a 2-fold change and 108 genes that were down-regulated by  $\beta$ -estradiol in Mg-63 cells, compared to untreated cells. TE-85 cells showed just one gene down-regulated when compared to non-treated controls. There was one gene that was up-regulated and 2 genes that were down-regulated in Saos-2-treated cells compared to untreated cells. In MCF-7 control estrogen-responsive cells, 142 genes were found to be upregulated and 137 genes were found to be downregulated by  $\beta$ -estradiol compared to untreated cells. These results are

consistent with the presence of estrogen receptor  $\alpha$  protein in the Mg-63 and MCF-7 cells and the very low levels of this receptor in the TE-85 and Saos-2 cells (Figure 3-3).



**Figure 3-9:Volcano plot of the fold-change of transcripts in osteosarcoma cells treated with  $\beta$ -estradiol compared to untreated control.**

Volcano plots were constructed by plotting  $-\log_{10}$  of the q-value on the y-axis, and the expression fold change between control and cells treated with 1 nM of  $\beta$ -estradiol on the x-axis. In the plots, each dot represents one gene, and control vs  $\beta$ -estradiol treatment was plotted for the cultured osteosarcoma cell lines and MCF-7 cells. Genes that pass the filtering of q-value < 0.05 are indicated in green on the plot, and those that do not are in red.

### 3.2.4.3.2 Differentially Expressed Estrogen Responsive Genes in Osteosarcoma Cells

From 262 gene found to be differentially expressed in the  $\beta$ -estradiol-treated Mg-63 cells, 36 genes were increased by over 2-fold ( $p < 0.05$ ,  $q < 0.05$ ) and 19 genes were decreased over 2-fold change ( $p < 0.05$ ,  $q < 0.05$ ) in the  $\beta$ -estradiol-treated cells compared to the untreated control (Table 3-5).

12 mRNAs, including DKK3 (Dickkopf-related protein 3), XAF1 (XIAP-associated factor 1), LSP1 (Lymphocyte-specific protein 1), CREB5 (cAMP responsive element binding protein 5), MMP2 (Matrix metalloproteinase 2), COL1A2 (Collagen type I alpha 2 chain), FSTL1 (Follistatin-like 1), IFI44L (Interferon-induced protein 44 like), DCN (Decorin), IFI16 (Gamma-interferon-inducible protein 16), BLID (BH3-like motif containing, cell death inducer) and CALD1 (Caldesmon 1), showed the highest fold changed by over 9-fold in expression in the Mg-63 cells treated with  $\beta$ -estradiol compared to untreated control.

7 mRNAs, COL3A1 (Collagen type III alpha 1 chain), VIM (Vimentin), MX1 (MX dynamin like GTPase 1), IFI27 (Interferon alpha inducible protein 27), GSDMD (Gasdermin D), OAS2 (2'-5'-oligoadenylate synthetase 2) and S100A4 (S100 calcium binding protein A4), showed over 8-fold increased expression in the  $\beta$ -estradiol-treated Mg-63 cells.

The levels of 13 mRNAs including ALDH3A1 (Aldehyde dehydrogenase 3 family member A1), FBN1 (Fibrillin 1), PARP10 (Poly(ADP-ribose) polymerase family



member 10), TMBIM1 (Transmembrane BAX inhibitor motif containing 1), TGFBI (Transforming growth factor beta induced), IFI6 (Interferon alpha inducible protein 6), CTSZ (Cathepsin Z), ANXA1 (Annexin A1), COL1A1 (Collagen type I alpha 1 chain), TRAM2 (Translocation associated membrane protein 2), SPOCK1 (SPARC/osteonectin, cwcv and kazal like domains proteoglycan 1), FKBP1 (FK506 binding protein 10) and EMP1 (Epithelial membrane protein 1) showed an increase in level of over 5-fold change in the  $\beta$ -estradiol treated Mg-63 cells compared to the untreated control.

The levels of 4 mRNAs, S100A2 (S100 calcium binding protein A2), EPB41L2 (Erythrocyte membrane protein band 4.1 like 2), LTBP4 (Latent transforming growth factor beta binding protein 4) and SLC16A3 (Solute carrier family 16 (monocarboxylic acid transporters), member 3) were upregulated by over 3-fold change in the  $\beta$ -estradiol-treated Mg-63 cells compared to the untreated control.

The levels of 5 mRNAs, FXYP3 (FXYP domain containing ion transport regulator 3), MLPH (Melanophilin), LLGL2 (LLGL2, scribble cell polarity complex component) ESR1 (Estrogen receptor 1) and ESRP2 (Epithelial splicing regulatory protein 2) were decreased by over 8-fold change in the  $\beta$ -estradiol treated Mg-63 cells compared to the untreated control.

The levels of 9 mRNAs, KRT19 (Keratin 19), CELSR2 (Cadherin EGF LAG seven-pass G-type receptor 2), GREB1 (Growth regulation by estrogen in breast cancer 1), MAL2 (Mal, T-cell differentiation protein 2), NPY1R (Neuropeptide Y receptor type 1), KRT8 (Keratin 8), TFAP2C (Transcription factor AP-2 gamma), GFRA1 (GDNF family receptor alpha 1) and TPD52L1 (Tumor protein D52 like 1) were decreased by

over 5-fold change in the  $\beta$ -estradiol treated Mg-63 cells compared to the untreated control.

The levels of 5 mRNAs including TMEM64 (Transmembrane protein 64), TRIM37 (Tripartite motif containing 37), NFKBIA (NFKB inhibitor alpha), XBP1 (X-box binding protein 1), RUNX2 (Runt related transcription factor 2) were decreased by over 2-fold change in the  $\beta$ -estradiol treated Mg-63 cells compared to the untreated control.

Of these 55 differentially-expressed genes, 51 were significantly changed in RNA-sequencing data ( $p < 0.05$ ), but 4 mRNAs, S100A2, RUNX2, XBP1, NFKB1A were differentially expressed between treated and untreated control, but were not significant in that data ( $p = 0.84$ ,  $p = 0.88$ ,  $p = 0.46$ , and  $p = 0.51$  respectively).

**Table 3-5: Fold changes of the top 55 identified up and down regulated mRNA between control and Estradiol treated Mg-63 cells from RNA-seq generated by TGAC.**

	Gene name	Gene symbol	FPKM of Control	FPKM of Treated	Fold Change	p-value	q-Value
1	Dickkopf-related protein 3	DKK3	0.82	784.73	9.91	0.0066	0.0065
2	XIAP-associated factor 1	XAF1	0.10	92.32	9.87	0.0066	0.0065
3	Lymphocyte-specific protein 1	LSP1	0.05	48.54	9.83	0.0066	0.0065
4	cAMP responsive element binding protein 5	CREB5	0.08	76.55	9.82	0.0066	0.0065
5	Matrix metalloproteinase 2	MMP2	1.26	1073.33	9.73	0.0482	0.0481
6	Collagen type I alpha 2 chain	COL1A2	1.56	1295.90	9.70	0.0066	0.0065
7	Follistatin like 1	FSTL1	1.15	897.09	9.61	0.0066	0.0065
8	Interferon induced protein 44 like	IFI44L	0.41	323.98	9.61	0.0298	0.0298

9	Decorin	DCN	0.17	130.86	9.60	0.0066	0.0065
10	Gamma-interferon-inducible protein 16	IFI16	0.27	186.49	9.41	0.0066	0.0065
11	BH3-like motif containing, cell death inducer	BLID	0.09	61.24	9.35	0.0298	0.0298
12	Caldesmon 1	CALD1	0.13	69.11	9.04	0.0066	0.0065
13	Collagen type III alpha 1 chain	COL3A1	2.78	1335.38	8.91	0.0164	0.0163
14	Vimentin	VIM	6.05	2730.81	8.82	0.0066	0.0065
15	MX dynamin like GTPase 1	MX1	1.78	713.49	8.64	0.0066	0.0065
16	Interferon alpha inducible protein 27	IFI27	4.37	1618.77	8.53	0.0066	0.0065
17	Gasdermin D	GSDMD	0.47	145.74	8.28	0.0066	0.0065
18	2'-5'-oligoadenylate synthetase 2	OAS2	0.69	199.89	8.17	0.0429	0.0428
19	<b>S100 calcium binding protein A4</b>	<b>S100A4</b>	<b>2.55</b>	<b>691.06</b>	<b>8.08</b>	<b>0.0043</b>	<b>0.017</b>
20	Aldehyde dehydrogenase 3 family member A1	ALDH3A1	2.83	555.70	7.62	0.0119	0.0119
21	Fibrillin 1	FBN1	1.66	257.19	7.27	0.0066	0.0065
22	Poly(ADP-ribose) polymerase family member 10	PARP10	1.51	147.67	6.61	0.0066	0.0065
23	Transmembrane BAX inhibitor motif containing 1	TMBIM1	1.27	123.18	6.60	0.0352	0.0351
24	Transforming growth factor beta induced	TGFBI	3.81	349.70	6.52	0.0066	0.0065
25	Interferon alpha inducible protein 6	IFI6	22.29	2019.69	6.50	0.0298	0.0298
26	Cathepsin Z	CTSZ	2.04	183.08	6.48	0.0119	0.0119
27	Annexin A1	ANXA1	3.21	282.05	6.46	0.0164	0.0163
28	Collagen type I alpha 1 chain	COL1A1	11.61	733.64	5.98	0.0066	0.0065
29	Translocation associated membrane protein 2	TRAM2	7.75	454.53	5.87	0.0268	0.0268
30	SPARC/osteonectin, cwcv and kazal like domains proteoglycan 1	SPOCK1	3.93	225.96	5.85	0.0429	0.0428
31	FK506 binding protein 10	FKBP10	3.72	193.00	5.70	0.0164	0.0163
32	Epithelial membrane protein 1	EMP1	4.18	145.36	5.12	0.0066	0.0065
33	<b>S100 calcium binding protein A2</b>	<b>S100A2</b>	<b>0.38</b>	<b>5.65</b>	<b>3.88</b>	<b>0.17</b>	<b>0.84</b>
34	Erythrocyte membrane protein band 4.1 like 2	EPB41L2	14.37	198.02	3.78	0.0482	0.0481

<b>35</b>	Latent transforming growth factor beta binding protein 4	LTBP4	20.97	265.43	3.66	0.0066	0.0065
<b>36</b>	Solute carrier family 16 (monocarboxylic acid transporters), member 3	SLC16A3	10.33	112.61	3.45	0.0429	0.0428
<b>37</b>	<b>Runt related transcription factor 2</b>	<b>RUNX2</b>	<b>1.55</b>	<b>0.39</b>	<b>-2</b>	<b>0.29</b>	<b>0.88</b>
<b>38</b>	<b>X-box binding protein 1</b>	<b>XBP1</b>	<b>1503.4</b>	<b>120.15</b>	<b>-3.64</b>	<b>0.021</b>	<b>0.46</b>
<b>39</b>	<b>NFKB inhibitor alpha</b>	<b>NFKBIA</b>	<b>187.63</b>	<b>12.8</b>	<b>-3.87</b>	<b>0.025</b>	<b>0.51</b>
<b>40</b>	Tripartite motif containing 37	TRIM37	290.90	14.51	-4.33	0.0429	0.0428
<b>41</b>	Transmembrane protein 64	TMEM64	480.05	17.39	-4.79	0.0268	0.0268
<b>42</b>	Tumor protein D52 like 1	TPD52L1	361.19	10.77	-5.07	0.0066	0.0065
<b>43</b>	GDNF family receptor alpha 1	GFRA1	116.38	1.74	-6.07	0.0298	0.0298
<b>44</b>	Transcription factor AP-2 gamma	TFAP2C	140.81	2.08	-6.08	0.0298	0.0298
<b>45</b>	Keratin 8	KRT8	2401.25	32.45	-6.21	0.0268	0.0268
<b>46</b>	Neuropeptide Y receptor type 1	NPY1R	142.49	1.70	-6.39	0.0268	0.0268
<b>47</b>	Mal, T-cell differentiation protein 2	MAL2	151.01	1.52	-6.63	0.0352	0.0351
<b>48</b>	Growth regulation by estrogen in breast cancer 1	GREB1	154.39	1.47	-6.72	0.0066	0.0065
<b>49</b>	Cadherin EGF LAG seven-pass G-type receptor 2	CELSR2	238.68	1.72	-7.11	0.0268	0.0268
<b>50</b>	Keratin 19	KRT19	1020.44	6.39	-7.32	0.0066	0.0065
<b>51</b>	Epithelial splicing regulatory protein 2	ESRP2	94.99	0.28	-8.42	0.0298	0.0298
<b>52</b>	Estrogen receptor 1	ESR1	133.69	0.38	-8.46	0.0237	0.0236
<b>53</b>	LLGL2, scribble cell polarity complex component	LLGL2	204.89	0.58	-8.47	0.0066	0.0065
<b>54</b>	Melanophilin	MLPH	473.11	1.14	-8.69	0.0298	0.0298
<b>55</b>	FXRD domain containing ion transport regulator 3	FXRD3	561.10	1.04	-9.08	0.0202	0.0201

FPKM is Fragments Per Kilobase of transcript per Million mapped reads. The ratio  $\geq 2$  fold changed for up-downregulated genes by  $\beta$ -estradiol with q-value  $< 0.05$  was significant. Bold data represent bone-related genes.

### **3.2.4.3.3 Identification of Common Estrogen Responsive Genes Between Mg-63 and MCF-7 cells:**

Interrelation analyses between differentially expressed genes were performed between Mg-63 and MCF-7 cells to identify common estrogen responsive genes using intractiVenn web tool (Heberle et al., 2015). Interestingly, 181 genes, out of 262 that were differentially expressed in the Mg-63 cells, were found to be also differentially expressed in the MCF-7 cells. Therefore, this 65% of identified  $\beta$ -estradiol-responsive genes in Mg-63 cells were the same as those in the ER-positive control cells, MCF-7, suggesting that these are common estrogen responsive genes. The remaining 81 genes differentially expressed in the Mg-63 cells were not differentially expressed by  $\beta$ -estradiol in the MCF-7 cells, possibly reflecting the different origins of the cells, i.e. bone and breast cells (Table 3-6) and suggests that these 81 genes might be bone-specific, estrogen-responsive mRNAs.

**Table 3-6: Common estrogen responsive genes between Mg-63 and MCF-7 cells**

Cells	No of Genes	Genes
<b>Mg-63</b>	81	GABRE, SPARC, <b>MMP2</b> , <b>COL1A2</b> , TENM2, C4orf22, TNC, CACNA1A, FAP, ACSL4, DAPK1, VCAN, <b>FBN1</b> , ABCA8, CNN3, MXRA8, OSBPL3, DDX60, C9, <b>IFI6</b> , <b>ANXA1</b> , NFIX, ALDH1A2, PTPRM, <b>TRAM2</b> , <b>SPOCK1</b> , RIMS1, AKAP2, EYA4, CADPS, SEMA5A, S100A6, TGFBI1, C1orf228, CCDC88A, BTN3A2, PDE4D, PSIP1, EFCAB13, NFASC, AUTS2, <b>EPB41L2</b> , IL15RA, <b>SLC16A3</b> , GALT, PALM2, AKAP2, RP11290H9.2, RP1195F19.29, FGF5, KCNE5, DAB2, PALM2, ITGB3, IL11RA, LINC01515, PPP1R13B, PLXNA3, <b>TMEM64</b> , ST6GALNAC2, FAM84B, IGF2, RAPGEFL1, RAB17, <b>TFAP2C</b> , <b>NPY1R</b> , VIPR1, CDYL2, <b>MAL2</b> , <b>GREB1</b> , RBM47, SLC7A2, <b>CELSR2</b> , CDH3, <b>KRT19</b> , SERPINA3, <b>ESRP2</b> , PKIB, INS-IGF2, INS, SERPINA4, SERPINA5
<b>Mg-63 and MCF-7</b>	181	MAGI2AS3, BNC2, RP11576C2.1, TXLNGY, <b>DKK3</b> , <b>XAF1</b> , <b>CREB5</b> , LAMA4, <b>FSTL1</b> , <b>IFI44L</b> , <b>DCN</b> , <b>IFI16</b> , <b>BLID</b> , TCF4, ADD2, <b>CALD1</b> , <b>COL3A1</b> , <b>VIM</b> , FCGRT, <b>MX1</b> , PIEZO2, MASP1, <b>IFI27</b> , APBB1, <b>GSDMD</b> , <b>OAS2</b> , ZEB1, PDE4B, C1R, C1RL, CDH11, RAB34, ZEB2, NLRC5, GPR162, P3H3, <b>ALDH3A1</b> , TPM2, PKNX2, PRG2, RP11872D17.8, SLC43A3, LIMS2, ADAMTSL1, LDB2, ABI3BP, NTM, COL14A1, MEF2C, SPON2, <b>PARP10</b> , ADAMTS10, <b>TMBIM1</b> , NCAM1, EFEMP2, <b>TGFBI</b> , <b>CTS2</b> , <b>EMP3</b> , MYOM2, NR2F1A1, TMEM173, LAMB1, SLC14A1, HOXB3, HOXB4, HOXB6, NAV1, TNFRSF14, <b>COL1A1</b> , BTN3A3, <b>FKBP10</b> , KCNMA1, HOXBAS1, HOXBAS3, TRO, APOL3, LPIN1, TSPAN32, UCHL1, COL16A1, DGKG, ETV5, <b>EMP1</b> , DDX60L, IQCJ, IQCJSCHIP1, SCHIP1, KIFC3, ROBO3, FGFR1, GSTM1, GSTM2, GSTM4, PLSCR1, FIP1L1, PDGFRA, ZNF532, <b>LTBP4</b> , SORBS2, ENOSF1, TM7SF2, VPS51, SLC25A29, ANKRD18A, RP11392E22.9, CCDC78, PCDHA1, PCDHA10, PCDHA11, PCDHA12, PCDHA13, PCDHA2, PCDHA3, PCDHA4, PCDHA5, PCDHA6, PCDHA7, PCDHA8, PCDHA9, PCDHAC1, PCDHAC2, TMEM132A, <b>TRIM37</b> , TMPRSS4, DTNA, TMC6, EPS8L1, ANK3, INPP5J, ISYNA1, MCF2L, TPD52L1, FER1L4, CNKSR1, MTUS1, FCHO1, PLEKHH1, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, SCUBE2, <b>GFRA1</b> , ADGRG1, ICA1, ATP2A3, <b>KRT8</b> , SLAIN1, LDLRAD4, CATSPERB, TC2N, HID1, SERPINA1, TTC39A, GALNT6, NRCAM, PTPN6, LAD1, TNNT2, MYB, AC011513.3, CEACAM21, CEACAM5, CEACAM6, PRLR, C19orf33, CTB102L5.4, SPINT2, <b>ESR1</b> , <b>LLGL2</b> , ATP2C2, <b>MLPH</b> , CDH1, <b>FXYD3</b> , <b>LSP1</b> , DMKN

Out of 262 differentially-changed genes upon  $\beta$ -estradiol treatment of Mg-63, 181 genes were also differentially expressed in MCF-7 cells. 81 genes were differentially expressed in the Mg-63 cells but not in the MCF-7 cells and thus may be bone-specific estrogen responsive mRNAs. Bold genes are those among the list of 55 Mg-63 genes altered by 2-fold or greater.

### 3.2.4.3.4 Functional Analysis of the Identified Differentially Expressed mRNAs

Functional analysis of the 55 identified differentially-expressed mRNAs in treated Mg-63 compared to the control is summarized in Table 3-7. The results show that some of these genes are known to be involved in the regulation of bone remodelling, such as DKK3, XAF1, MMP2, COL1A2, IFI44L, IFI16, VIM, MX1, PARP10, TGFBI, CTSZ, COL1A1, TRAM2, TMEM64, KRT8, CELSR2 and ESR1. Estrogen is shown to be involved in the differential expression of BLID, ANXA1, CALD1, CTSZ, TRAM2, EMP1, TFAP2C, GREB1, and ESR1. Others have been suggested to be changed in osteoarthritis (OA) like DKK3, FSTL1, CALD1 and COL3A1. Another group of genes (XAF1, IFI16, BLID, MX1, IFI27, GSDMD, TMBIMI, and GFRA1) may be involved in the apoptosis signalling pathway.

DKK3 (Dickkopf-WNT signalling pathway inhibitor 3), is a secreted protein which has been reported to be associated with cancer development (Kawauchi et al., 2012) (Kurose et al., 2004, Veeck et al., 2008, Yue et al., 2008). The increased mRNA and protein levels of DKK3 in human osteoarthritis cartilage, synovial tissue and synovial fluid and may have a positive effect on cartilage integrity by preventing proteoglycan loss and helping to restore osteoarthritis-relevant signalling pathway activity (Snelling et al., 2016). DKK3 has been reported to be able to inhibit invasion and motility of Saos-2 osteosarcoma cells through modulation of the Wnt-beta-catenin pathway (Hoang et al., 2004).

XAF1 (X-linked inhibitor of apoptosis associated factor 1) protein has been reported to be involved in the progress of apoptosis signalling pathway (Liston et al.,

2001), to inhibit cell proliferation and induce cell apoptosis in human lung adenocarcinoma cells (Chen et al., 2011) and to inhibit TNF-induced NF- $\kappa$ B activation (Lin et al., 2016). MMP2 (matrix metalloproteinase-2) is a protein that is involved in the breakdown of extracellular matrix (ECM) in normal physiological processes (Clark et al., 2007). MMP-2 plays a direct role in early skeletal development and bone cell growth (Mosig et al., 2007). Suppression of MMP-2 in osteoblastic murine increases osteopontin (OPN) and bone sialoprotein (BSP) expression and thereby regulates bone homeostasis (Mosig and Martignetti, 2013).

COL1A2 (Collagen type I alpha 2 chain) is found in most connective tissue and is a key structural component of the extracellular matrix (Cole, 1994) and involved in several cancers (Ibanez de Caceres et al., 2006, Hayashi et al., 2014, Bonazzi et al., 2011). Mutation of COL1A2 has been found to be associated with low BMD and fractures in Icelandic people (Styrkarsdottir et al., 2016). Suppression of COL1A2 may be associated with a reduced risk of developing osteoporosis by decrease the ability of osteogenesis (Jiang and Hao, 2016). Collagen type I alpha 1 chain (COL1A1) is an extra-cellular matrix protein (Kaneto et al., 2014) which was inactivated in most tissues, but found to be activated during osteoblast differentiation (Rossert et al., 1995, Stover et al.). Also, COL1A1 can be used as markers of increasing maturation within the osteoblast lineage (Kalajzic et al., 2005, Glass Ii et al., 2005).

VIM (vimentin) is a member of the intermediate filament protein family and is considered to be a molecular marker of mesenchymal cells (Lian et al., 2012b). TGF- $\beta$  increases VIM expression in osteoblast cells, which leads to suppression of ATF4-



dependent Ocn transcription and inhibit the osteoblast differentiation (Lian et al., 2012b). VIM also inhibits endogenous osteocalcin transcription and osteoblast differentiation, and inhibits the expression of osteoblast marker genes (Lian et al., 2009).

Keratin (KRT8) is the largest family of intermediate filament in which play role in autoimmune diseases (Toivola et al., 2015) and rheumatoid arthritis (Wang et al., 2015). It has been reported that suppression of KRT8 could cause osteopenia through the reduction of osteoblast differentiation in normal mice by inhibiting NF- $\kappa$ B and Wnt- $\beta$ -catenin signals in the bones (Le Henaff et al., 2016).

Cathepsin Z (CTSZ) is a lysosomal cysteine proteinase and member of the peptidase C1 family (Santamaria et al., 1998). and present in the cells of immune system, predominantly monocytes, macrophages and dendritic cells (Kos et al., 2005). CTSZ enzyme was found also to be secreted from non-hematopoietic bone marrow cells and found predominantly on the surface of primary human osteoblasts and suppression of CTSX in osteoblasts resulted in an enhanced cellular adhesive interaction between hematopoietic stem and progenitor cells to osteoblasts (HSPCs) (Staudt et al., 2010). The expression of cathepsin B, L and Z were downregulated during differentiation of myeloid-derived suppressor cells (MDSCs) from macrophage to osteoclast in mice bearing highly metastatic tumours. This might inhibit osteoclastogenesis by decreasing the fusion of osteoclast precursors through cleavage of proteins needed for membrane fusion (Edgington-Mitchell et al., 2015).

ANXA1 is a member of the annexin family that play as a mediator of glucocorticoid action in the inflammation and under control of hormone released from

anterior pituitary hormone release (John et al., 2004). Positive effects of  $17\beta$ -estradiol on the expression of ANXA1 mRNA in which an ovariectomised rat produces a decreased level of ANXA1 (Davies et al., 2007). Overall, the result demonstrated that identified differentially expressed genes may be involved in bone remodelling once the level of  $\beta$ -estradiol is changed in the culture osteosarcoma cell line, Mg-63.

**Table 3-7: The biological role of the 55 identified differentially expressed genes**

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>DKK3</b>	Dickkopf-WNT signalling pathway inhibitor 3	9.91	11p15.3	This gene encodes a protein that is a member of the dickkopf family. The secreted protein contains two cysteine rich regions and is involved in embryonic development through its interactions with the Wnt signaling pathway.	Wnt signalling pathway (GO:0016055)	calcium ion binding (GO:0005509)	extracellular space (GO:0005615)	(Snelling et al., 2016, Hoang et al., 2004)
<b>XAF1</b>	X-linked inhibitor of apoptosis-associated factor 1	9.87	17p13.1	This gene encodes a protein which binds to and counteracts the inhibitory effect of a member of the IAP (inhibitor of apoptosis) protein family affecting the progress of the apoptosis signalling pathway	apoptotic process (GO:0006915)	zinc ion binding (GO:0008270)	nucleus (GO:0005634)	(Lin et al., 2016)
<b>LSP1</b>	lymphocyte-specific protein 1	9.83	11p15.5	This gene encodes an intracellular F-actin binding protein. The protein is expressed in lymphocytes, neutrophils, macrophages, and endothelium and may regulate neutrophil motility, adhesion to fibrinogen matrix proteins, and transendothelial migration	movement of cell or subcellular component (GO:0006928)	actin binding (GO:0003779)	plasma membrane (GO:0005886)	(Hwang et al., 2015)
<b>CREB5</b>	cAMP responsive element binding protein 5	9.82	7p15.1	The product of this gene belongs to the CRE (cAMP response element)-binding protein family. Members of this family contain zinc-finger and bZIP DNA-binding domains. The encoded protein specifically binds to CRE as a homodimer or a heterodimer with c-Jun or CRE-BP1, and functions as a CRE-dependent trans-activator.	regulation of transcription, DNA-templated (GO:0045893)	DNA binding (GO:0003677)	nucleus (GO:0005634)	(Qi and Ding, 2014)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>MMP2</b>	matrix metalloproteinase 2	9.73	16q12.2	This gene is a member of the matrix metalloproteinase (MMP) gene family, that are zinc-dependent enzymes capable of cleaving components of the extracellular matrix and molecules involved in signal transduction. The protein encoded by this gene is a gelatinase A, type IV collagenase, that contains three fibronectin type II repeats in its catalytic site that allow binding of denatured type IV and V collagen and elastin This may be involved in multiple pathways including roles in the nervous system, endometrial menstrual breakdown, regulation of vascularization, and metastasis.	bone trabecula formation (GO:0060346)	metalloendopeptidase activity (GO:0004222)	extracellular space (GO:0005615)	(Mosig and Martignetti, 2013, Mosig et al., 2007)
<b>COL1A2</b>	collagen type I alpha 2 chain	9.70	7q21.3	This gene encodes the pro-alpha2 chain of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain.	skeletal system development (GO:0001501)	extracellular matrix structural constituent (GO:0005201)	extracellular space (GO:0005615)	(Jiang and Hao, 2016, Stykarsdottir et al., 2016)
<b>FSTL1</b>	follistatin like 1	9.61	3q13.33	This gene encodes a protein with similarity to follistatin, an activin-binding protein.	bone morphogenetic protein signalling pathway (GO:0030509)	calcium ion binding (GO:0005509)	extracellular space (GO:0005615)	(Ni et al., 2015)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>IFI44L</b>	interferon induced protein 44 like	9.61	1p31.1	Exhibits a low antiviral activity against hepatitis C virus.	defense response to virus (GO:0051607)	GTP binding (GO:0005525)	cytoplasm (GO:0005737)	(WoECKel et al., 2012a)
<b>DCN</b>	decorin	9.60	12q21.33	This gene encodes a member of the small leucine-rich proteoglycan family of proteins. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed to generate the mature protein. This protein plays a role in collagen fibril assembly. Binding of this protein to multiple cell surface receptors mediates its role in tumor suppression, including a stimulatory effect on autophagy and inflammation and an inhibitory effect on angiogenesis and tumorigenesis.	skeletal muscle tissue development (GO:0007519)	collagen binding (GO:0005518)	extracellular region (GO:0005518)	(Goetsch and Niesler, 2016, Bi et al., 2005)
<b>IFI16</b>	Gamma-interferon-inducible protein 16	9.41	1q23.1	This gene encodes a member of the HIN-200 (hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats) family of cytokines. The encoded protein contains domains involved in DNA binding, transcriptional regulation, and protein-protein interactions. The protein localizes to the nucleoplasm and nucleoli, and interacts with p53 and retinoblastoma-1.	autophagy (GO:0006914)	RNA binding (GO:0003723)	membrane (GO:0016020)	(Zhang et al., 2007)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>BLID</b>	BH3-like motif containing, cell death inducer	9.35	11q24.1	This gene encodes a BH3-like motif containing protein involved in cell death. The encoded protein may induce apoptosis in a caspase-dependent manner.	apoptotic process (GO:0006915)	-	cytoplasm (GO:0005737)	(Li et al., 2014)
<b>CALD1</b>	caldesmon 1	9.04	7q33	This gene encodes a calmodulin- and actin-binding protein that plays an essential role in the regulation of smooth muscle and nonmuscle contraction. The conserved domain of this protein possesses the binding activities to Ca(2+)-calmodulin, actin, tropomyosin, myosin, and phospholipids. This protein is a potent inhibitor of the actin-tropomyosin activated myosin MgATPase, and serves as a mediating factor for Ca(2+)-dependent inhibition of smooth muscle contraction.	muscle contraction (GO:0006936)	myosin binding (GO:0017022)	cytoskeleton (GO:0005856)	(Mayanagi and Sobue, 2011, Zhang et al., 2012, De Marchi et al., 2016)
<b>COL3A1</b>	collagen type III alpha 1 chain	8.91	2q32.2	This gene encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues such as skin, lung, uterus, intestine and the vascular system, frequently in association with type I collagen. Mutations in this gene are associated with Ehlers-Danlos syndrome types IV, and with aortic and arterial aneurysms.	skeletal system development (GO:0001501)	extracellular matrix structural constituent (GO:0005201)	extracellular region (GO:0005518)	(Chou et al., 2013)
<b>VIM</b>	vimentin	8.82	10p13	This gene encodes a type III intermediate filament protein. Intermediate filaments, along with microtubules and actin microfilaments, make up the cytoskeleton. The encoded protein is responsible for maintaining cell shape and	movement of cell or subcellular component	structural constituent of cytoskeleton	cytoplasm (GO:0005737)	(Lian et al., 2009, Lian et

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
				integrity of the cytoplasm, and stabilizing cytoskeletal interactions. This protein is involved in neuritogenesis and cholesterol transport and functions as an organizer of a number of other critical proteins involved in cell attachment, migration, and signaling.	actin filament organization (GO:0006928)			al., 2012b)
<b>MX1</b>	MX dynamin like GTPase 1	8.64	21q22.3	This gene encodes a guanosine triphosphate (GTP)-metabolizing protein that participates in the cellular antiviral response. The encoded protein is induced by type I and type II interferons and antagonizes the replication process of several different RNA and DNA viruses.	apoptotic process (GO:0006915)	identical protein binding (GO:0042802)	cytoplasm (GO:0005737)	(Park et al., 2012)
<b>IFI27</b>	interferon alpha inducible protein 27	8.53	14q32.12	Promotes cell death. Mediates IFN-induced apoptosis characterized by a rapid and robust release of cytochrome C from the mitochondria and activation of BAX and caspases 2, 3, 6, 8 and 9.	apoptotic signalling pathway (GO:0097190)	RNA polymerase II activating transcription factor binding (GO:0001102)	nucleus (GO:0005634)	(Gytz et al., 2017)
<b>GSDMD</b>	gasdermin D	8.28	8q24.3	Gasdermin D is a member of the gasdermin family. Members of this family appear to play a role in regulation of epithelial proliferation. Gasdermin D has been suggested to act as a tumor suppressor.	pyroptosis (GO:0070269)	phosphatidic acid binding (GO:0070300)	plasma membrane (GO:0005886)	(Sborgi et al., 2016)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>OAS2</b>	2'-5'-oligoadenylate synthetase 2	8.17	12q24.13	This gene encodes a member of the 2-5A synthetase family, essential proteins involved in the innate immune response to viral infection. The encoded protein is induced by interferons and uses adenosine triphosphate in 2'-specific nucleotidyl transfer reactions to synthesize 2',5'-oligoadenylates (2-5As). These molecules activate latent RNase L, which results in viral RNA degradation and the inhibition of viral replication.	type I interferon signaling pathway (GO:0060337)	metal ion binding (GO:0046872)	cytoplasm (GO:0005737)	(WoECKel et al., 2012b)
<b>S100A4</b>	S100 calcium binding protein A4	<b>8.08</b>	1q21.3	The protein encoded by this gene is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. This protein may function in motility, invasion, and tubulin polymerization.	positive regulation of I-kappaB kinase/NF-kappaB signalling (GO:0043123)	calcium ion binding (GO:0005509)	extracellular space (GO:0005615)	(Erlandsson et al., 2013)
<b>ALDH3A1</b>	aldehyde dehydrogenase 3 family member A1	7.62	17p11.2	Aldehyde dehydrogenases oxidize various aldehydes to the corresponding acids. They are involved in the detoxification of alcohol-derived acetaldehyde and in the metabolism of corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation. The enzyme encoded by this gene forms a cytoplasmic homodimer that preferentially	response to hypoxia (GO:0001666)	3-chloroallyl aldehyde dehydrogenase activity (GO:0004028)	extracellular space (GO:0005615)	(Wu et al., 2016, Yan et al., 2014, Gasparotto et



Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
				oxidizes aromatic and medium-chain (6 carbons or more) saturated and unsaturated aldehyde substrates. It is thought to promote resistance to UV and 4-hydroxy-2-nonenal-induced oxidative damage in the cornea.				al., 2012)
<b>FBN1</b>	fibrillin 1	7.27	15q21.1	This gene encodes a member of the fibrillin family of proteins. The encoded preproprotein is proteolytically processed to generate two proteins including the extracellular matrix component fibrillin-1 and the protein hormone asprosin. Fibrillin-1 is an extracellular matrix glycoprotein that serves as a structural component of calcium-binding microfibrils.	cellular response to insulin-like growth factor stimulus (GO:1990314)	calcium ion binding (GO:0005509)	extracellular region (GO:0005518)	(Smaldone et al., 2016)
<b>PARP10</b>	poly(ADP-ribose) polymerase family member 10	6.61	8q24.3	Poly(ADP-ribose) polymerases (PARPs), such as PARP10, regulate gene transcription by altering chromatin organization by adding ADP-ribose to histones. PARPs can also function as transcriptional cofactors	negative regulation of NF-kappaB transcription factor activity (GO:0032088)	NAD+ ADP-ribosyltransferase activity (GO:0003950)	nucleus (GO:0005634)	(Kaufmann et al., 2015, Verheugd et al., 2013)
<b>TMBIM1</b>	transmembrane BAX inhibitor motif containing 1	6.60	2q35	Negatively regulates aortic matrix metalloproteinase-9 (MMP9) production and may play a protective role in vascular remodeling.	negative regulation of catalytic activity	death receptor binding (GO:0005123)	lysosomal membrane (GO:0005765)	(Shukla et al., 2011)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
					(GO:0043086)			
<b>TGFBI</b>	transforming growth factor beta induced	6.52	5q31.1	This gene encodes an RGD-containing protein that binds to type I, II and IV collagens. The RGD motif is found in many extracellular matrix proteins modulating cell adhesion and serves as a ligand recognition sequence for several integrins. This protein plays a role in cell-collagen interactions and may be involved in endochondrial bone formation in cartilage. The protein is induced by transforming growth factor-beta and acts to inhibit cell adhesion.	cell adhesion (GO:0007155)	collagen binding (GO:0005518)	plasma membrane (GO:0005886)	(Bonewald and Mundy, 1990, Lee et al., 2015)
<b>IFI6</b>	interferon alpha inducible protein 6	6.50	1p35.3	This gene was first identified as one of the many genes induced by interferon. The encoded protein may play a critical role in the regulation of apoptosis. A minisatellite that consists of 26 repeats of a 12 nucleotide repeating element resembling the mammalian splice donor consensus sequence begins near the end of the second exon.	type I interferon signaling pathway (GO:0060337)	protein binding (GO:0008234)	plasma membrane (GO:0005886)	(Song et al., 2015)
<b>CTSZ</b>	Cathepsin Z	6.48	20q13.32	The protein encoded by this gene is a lysosomal cysteine proteinase and member of the peptidase C1 family. It exhibits both carboxy-monopeptidase and carboxy-dipeptidase activities. The encoded protein has also been known as cathepsin X and cathepsin P. This gene is expressed ubiquitously in cancer cell lines and primary tumors and, like other	proteolysis (GO:0006508)	protein binding (GO:0008234)	lysosome (GO:0005764)	(Liu et al., 2014a)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
				members of this family, may be involved in tumorigenesis.				
<b>ANXA1</b>	annexin A1	6.46	9q21.13	This gene encodes a membrane-localized protein that binds phospholipids. This protein inhibits phospholipase A2 and has anti-inflammatory activity. Loss of function or expression of this gene has been detected in multiple tumors.	response to estradiol (GO:0032355)	phospholipid binding (GO:0005543)	nucleus (GO:0005634)	(Ang et al., 2009)
<b>COL1A1</b>	collagen type I alpha 1 chain	5.98	17q21.33	This gene encodes the pro-alpha1 chains of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon. Mutations in this gene are associated with osteogenesis imperfecta types I-IV, Ehlers-Danlos syndrome type VIIA, Ehlers-Danlos syndrome Classical type, Caffey Disease and idiopathic osteoporosis.	osteoblast differentiation (GO:0001649)	metal ion binding (GO:0050900)	extracellular space (GO:0005615)	(Rosser et al., 1995)
<b>TRAM2</b>	translocation associated membrane protein 2	5.87	6p12.2	TRAM2 is a component of the translocon, a gated macromolecular channel that controls the posttranslational processing of nascent secretory and membrane proteins at the endoplasmic reticulum (ER) membrane.	collagen biosynthetic process (GO:0032964)	-	integral component of membrane (GO:0016021)	(Liu et al., 2014a)
<b>SPOCK1</b>	SPARC/osteonectin, cwcv and kazal like	5.85	5q31.2	This gene encodes the protein core of a seminal plasma proteoglycan containing chondroitin- and heparan-sulfate chains. The protein's function is unknown, although similarity to thyropin-type	regulation of cell growth(G	calcium ion binding	cytoplasm (GO:0005737)	(Chen et al., 2016)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
	domains proteoglycan 1			cysteine protease-inhibitors suggests its function may be related to protease inhibition.	O:0001558)	(GO:0005509)		
<b>FKBP10</b>	FK506 binding protein 10	5.70	17q21.2	The protein encoded by this gene belongs to the FKBP-type peptidyl-prolyl cis/trans isomerase (PPIase) family. This protein localizes to the endoplasmic reticulum and acts as a molecular chaperone. Alternatively, spliced variants encoding different isoforms have been reported, but their biological validity has not been determined	chaperone-mediated protein folding(GO:0061077)	calcium ion binding(GO:0005509)	cytoplasm (GO:0005737)	(Xu et al., 2017)
<b>EMP1</b>	epithelial membrane protein 1	5.12	12p13.1	SOS1 and Ras regulate epithelial tight junction formation in the human airway through EMP1	cell death (GO:0008219)	protein binding (GO:0008234)	plasma membrane (GO:0005886)	(Sun et al., 2014) (Sun et al., 2014)
<b>S100A2</b>	S100 calcium binding protein A2	<b>3.88</b>	1q21.3	The protein encoded by this gene is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation.	endothelial cell migration (GO:0043542)	calcium ion binding(GO:0005509)	cellular component (GO:0005575)	(Balmain et al., 2003)
<b>EPB41L2</b>	erythrocyte membrane protein band 4.1 like 2	3.78	6q23.1-q23.2	Required for dynein-dynactin complex and NUMA1 recruitment at the mitotic cell cortex during anaphase	cell cycle(GO:0007049)	actin binding(GO:0003779)	cytoplasm (GO:0005737)	(Lu et al., 2004)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>LTBP4</b>	latent transforming growth factor beta binding protein 4	3.66	19q13.2	The protein encoded by this gene binds transforming growth factor beta (TGFB) as it is secreted and targeted to the extracellular matrix. TGFB is biologically latent after secretion and insertion into the extracellular matrix, and sheds TGFB and other proteins upon activation. Defects in this gene may be a cause of cutis laxa and severe pulmonary, gastrointestinal, and urinary abnormalities	growth hormone secretion (GO:0030252)	transforming growth factor beta binding (GO:0050431)	extracellular exosome (GO:0070062)	(Lu et al., 2017)
<b>SLC16A3</b>	solute carrier family 16 (monocarboxylic acid transporters), member 3	3.45	17q25.3	Lactic acid and pyruvate transport across plasma membranes is catalyzed by members of the proton-linked monocarboxylate transporter (MCT) family, which has been designated solute carrier family-16. Each MCT appears to have slightly different substrate and inhibitor specificities and transport kinetics, which are related to the metabolic requirements of the tissues in which it is found.	monocarboxylic acid transport (GO:0015718)	monocarboxylic acid transmembrane transporter activity (GO:0008028)	membrane (GO:0016020)	(Tan et al., 2015)
<b>RUNX2</b>	Runt related transcription factor 2	-2	6p21.	This gene is a member of the RUNX family of transcription factors and encodes a nuclear protein with a Runt DNA-binding domain. This protein is essential for osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression. The protein can bind DNA both as a monomer or, with more affinity, as a subunit of a heterodimeric complex.	protein binding (GO:0005515)	osteoblast differentiation (GO:0001649)	cytoplasm (GO:0005737)	(Komori, 2010)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>XBP1</b>	X-box binding protein 1	<b>-3.64</b>	22q12.1; 22q12	This gene encodes a transcription factor that regulates MHC class II genes by binding to a promoter element referred to as an X box. This gene product is a bZIP protein, which was also identified as a cellular transcription factor that binds to an enhancer in the promoter of the T cell leukemia virus type 1 promoter. It may increase expression of viral proteins by acting as the DNA binding partner of a viral transactivator. It has been found that upon accumulation of unfolded proteins in the endoplasmic reticulum (ER), the mRNA of this gene is processed to an active form by an unconventional splicing mechanism that is mediated by the endonuclease inositol-requiring enzyme 1 (IRE1). The resulting loss of 26 nt from the spliced mRNA causes a frame-shift and an isoform XBP1(S), which is the functionally active transcription factor.	protein binding (GO:0005515)	endoplasmic reticulum unfolded protein response (GO:0030968)	cytoplasm (GO:0005737)	(Tohmonda et al., 2011)
<b>NFKBIA</b>	NFkB inhibitor alpha	<b>-3.87</b>	14q13.2	This gene encodes a member of the NF-kappa-B inhibitor family, which contain multiple ankyrin repeat domains. The encoded protein interacts with REL dimers to inhibit NF-kappa-B/REL complexes which are involved in inflammatory responses. The encoded protein moves between the cytoplasm and the nucleus via a nuclear localization signal and CRM1-mediated nuclear export.	NF-kappaB binding (GO:0051059)	negative regulation of NF-kappaB transcription factor activity (GO:0032088)	cytoplasm (GO:0005737)	(Novack, 2011)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>TRIM37</b>	tripartite motif containing 37	-4.33	17q22	This gene encodes a member of the tripartite motif (TRIM) family, whose members are involved in diverse cellular functions such as developmental patterning and oncogenesis. The TRIM motif includes zinc-binding domains, a RING finger region, a B-box motif and a coiled-coil domain. The RING finger and B-box domains chelate zinc and might be involved in protein-protein and/or protein-nucleic acid interactions.	negative regulation of NF-kappaB transcription factor activity (GO:0032088)	tumor necrosis factor receptor binding (GO:0005164)	cytoplasm (GO:0005737)	(Hu and Gan, 2017)
<b>TMEM64</b>	Transmembrane protein 64	-4.79	TMEM64	Positively regulates TNFSF11-induced osteoclast differentiation. Acts as a regulator of TNFSF11-mediated Ca <sup>2+</sup> signaling pathways via its interaction with SERCA2 which is critical for the TNFSF11-induced CREB1 activation and mitochondrial ROS generation necessary for proper osteoclast generation.	positive regulation of osteoclast differentiation (GO:0045672)	cytosolic calcium ion homeostasis	endoplasmic reticulum (GO:0005783)	(Kim et al., 2013)
<b>TPD52L1</b>	tumor protein D52 like 1	-5.07	6q22.31	This gene encodes a member of a family of proteins that contain coiled-coil domains and may form hetero- or homomers. The encoded protein is involved in cell proliferation and calcium signaling. It also interacts with the mitogen-activated protein kinase kinase kinase 5 (MAP3K5/ASK1) and positively regulates MAP3K5-induced apoptosis. Multiple alternatively spliced transcript variants have been observed.	positive regulation of apoptotic signalling pathway (GO:2001235)	protein binding (GO:0008234)	cytoplasm (GO:0005737)	(Sathasivam et al., 2001)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
<b>GFRA1</b>	GDNF family receptor alpha 1	-6.07	10q25.3	This gene encodes a member of the glial cell line-derived neurotrophic factor receptor (GDNFR) family of proteins. The encoded preproprotein is proteolytically processed to generate the mature receptor. Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) are two structurally related, potent neurotrophic factors that play key roles in the control of neuron survival and differentiation. This receptor is a glycosylphosphatidylinositol (GPI)-linked cell surface receptor for both GDNF and NTN, and mediates activation of the RET tyrosine kinase receptor.	MAPK cascade (GO:0000165)	receptor binding (GO:0005102)	plasma membrane (GO:0005886)	(Konishi et al., 2014)
<b>TFAP2C</b>	transcription factor AP-2 gamma	-6.08	20q13.31	The protein encoded by this gene is a sequence-specific DNA-binding transcription factor involved in the activation of several developmental genes. The encoded protein can act as either a homodimer or heterodimer with other family members and is induced during retinoic acid-mediated differentiation. It plays a role in the development of the eyes, face, body wall, limbs, and neural tube.	male gonad development (GO:0008584)	DNA binding (GO:0003677)	nucleus (GO:0005634)	(Orso et al., 2004) (Cyr et al., 2015)
<b>KRT8</b>	keratin 8	-6.21	12q13.13	This gene is a member of the type II keratin family clustered on the long arm of chromosome 12. Type I and type II keratins heteropolymerize to form intermediate-sized filaments in the cytoplasm of epithelial cells. The product of this gene typically dimerizes with keratin 18 to form	-	structural molecule activity (GO:0005198)	intermediate filament (GO:0005882)	(Wang et al., 2015, Le Henaff



Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
				an intermediate filament in simple single-layered epithelial cells. This protein plays a role in maintaining cellular structural integrity and also functions in signal transduction and cellular differentiation.				et al., 2016)
<b>NPY1R</b>	Neuropeptide Y receptor type 1	-6.39	4q32.2	This gene belongs to the G-protein-coupled receptor superfamily. The encoded transmembrane protein mediates the function of neuropeptide Y (NPY), a neurotransmitter, and peptide YY (PYY), a gastrointestinal hormone. The encoded receptor undergoes fast agonist-induced internalization through clathrin-coated pits and is subsequently recycled back to the cell membrane.	glucose metabolic process (GO:0006006)	pancreatic polypeptide receptor activity (GO:0001602)	plasma membrane (GO:0005886)	(Liu et al., 2015)
<b>MAL2</b>	mal, T-cell differentiation protein 2	-6.63	8q24.12	This gene encodes a multispan transmembrane protein belonging to the MAL proteolipid family. The protein is a component of lipid rafts and, in polarized cells, it primarily localizes to endosomal structures beneath the apical membrane. It is required for transcytosis, an intracellular transport pathway used to deliver membrane-bound proteins and exogenous cargos from the basolateral to the apical surface.	myelination (GO:0042552)	structural constituent of myelin sheath (GO:0019911)	extracellular exosome (GO:0070062)	(Marazuela et al., 2004, Marazuela and Alonso, 2004)
<b>GREB1</b>	growth regulation by estrogen in breast cancer 1	-6.72	2p25.1	This gene is an estrogen-responsive gene that is an early response gene in the estrogen receptor-regulated pathway. It is thought to play an important role in hormone-responsive tissues and cancer.	-	-	extracellular exosome (GO:0070062)	(Laviollette et al., 2014, Hodgkinson

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
								and Vanderhyden, 2014)
<b>CELSR2</b>	cadherin EGF LAG seven-pass G-type receptor 2	-7.11	1p13.3	The protein encoded by this gene is a member of the flamingo subfamily, part of the cadherin superfamily. The flamingo subfamily consists of nonclassic-type cadherins; a subpopulation that does not interact with catenins. The flamingo cadherins are located at the plasma membrane and have nine cadherin domains, seven epidermal growth factor-like repeats and two laminin A G-type repeats in their ectodomain. They also have seven transmembrane domains, a characteristic unique to this subfamily.	Wnt signalling pathway (GO:0016055)	calcium ion binding (GO:0005509)	plasma membrane (GO:0005886)	(Hu et al., 2011)
<b>KRT19</b>	keratin 19	-7.32	17q21.2	The protein encoded by this gene is a member of the keratin family. The keratins are intermediate filament proteins responsible for the structural integrity of epithelial cells and are subdivided into cytokeratins and hair keratins. The type I cytokeratins consist of acidic proteins which are arranged in pairs of heterotypic keratin chains. Unlike its related family members, this smallest known acidic cytokeratin is not paired with a basic cytokeratin in epithelial cells.	response to estrogen (GO:0043627)	structural constituent of cytoskeleton	plasma membrane (GO:0005886)	(Yao et al., 2016)
<b>ESRP2</b>	epithelial splicing regulatory protein 2	-8.42	16q22.1	mRNA splicing factor that regulates the formation of epithelial cell-specific isoforms. Specifically regulates the expression of FGFR2-IIIb, an epithelial cell-specific isoform of	regulation of RNA splicing	RNA binding (GO:0003723)	nucleus (GO:0005634)	(Ishii et al., 2014, Mizuta

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
				FGFR2. Also, regulates the splicing of CD44, CTNND1, ENAH, 3 transcripts that undergo changes in splicing during the epithelial-to-mesenchymal transition (EMT).	(GO:0043484)			ni et al., 2016)
<b>ESR1</b>	estrogen receptor 1	-8.46	6q25.1-q25.2	This gene encodes an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. Estrogen and its receptors are essential for sexual development and reproductive function, but also play a role in other tissues such as bone. Estrogen receptors are also involved in pathological processes including breast cancer, endometrial cancer, and osteoporosis.	cellular response to estradiol stimulus (GO:0071392)	estrogen receptor activity (GO:0030284)	nucleus (GO:0005634)	(Khalid and Krum, 2016)
<b>LLGL2</b>	LLGL2, scribble cell polarity complex component	-8.47	17q25.1	The lethal (2) giant larvae protein of Drosophila plays a role in asymmetric cell division, epithelial cell polarity, and cell migration. This human gene encodes a protein similar to lethal (2) giant larvae of Drosophila. In fly, the protein's ability to localize cell fate determinants is regulated by the atypical protein kinase C (aPKC). In human, this protein interacts with aPKC-containing complexes and is cortically localized in mitotic cells.	cell cycle (GO:0007049)	GTPase activator activity (GO:0005096)	cytoplasm (GO:0005737)	(Sripathy et al., 2011)
<b>MLPH</b>	melanophilin	-8.69	2q37.3	This gene encodes a member of the exophilin subfamily of Rab effector proteins. The protein forms a ternary complex with the small Ras-related GTPase Rab27A in its GTP-bound form	intracellular protein transport	metal ion binding (GO:0046872)	extracellular exosome	(Westbroek et al., 2012)

Gene Symbol	Gene Name	Fold Change	Chromosome Location	Description	Biological Process	Molecular Function	Cellular Component	Reference
				and the motor protein myosin Va. A similar protein complex in mouse functions to tether pigment-producing organelles called melanosomes to the actin cytoskeleton in melanocytes, and is required for visible pigmentation in the hair and skin.	(GO:0006886)		(GO:0070062)	
<b>FXYD3</b>	FXYD domain containing ion transport regulator 3	-9.08	19q13.12	This gene belongs to a small family of FXYP-domain containing regulators of Na <sup>+</sup> /K <sup>+</sup> ATPases which share a 35-amino acid signature sequence domain, beginning with the sequence PFXYP, and containing 7 invariant and 6 highly conserved amino acids. This gene encodes a cell membrane protein that may regulate the function of ion-pumps and ion-channels. This gene may also play a role in tumor progression.	ion transmembrane transport (GO:0034220)	sodium channel regulator activity (GO:0017080)	extracellular exosome (GO:0070062)	(Zhu et al., 2010)

### 3.2.4.3.5 Function Enrichment Analysis of the 262 Differentially-Expressed Genes in Mg-63 Cells

Enrichr, a web-accessible programme, was used for Gene Ontology (GO) enrichment analysis (Chen et al., 2013, Kuleshov et al., 2016). For such analysis, a  $p$  value  $< 0.05$  was set as the cut-off for selecting significantly-enriched functional GO terms. The results showed that the 262 estrogen responsive, differentially- expressed genes identified in Mg-63 were enriched in 128 GO biological process (Lewis et al.) terms. 14 out of the 128 GO BP terms enriched are presented in Table 3-8 while the reference of each term is summarized in Table 8-4 in the Appendix. Most of the 14 terms were related to either hormone response or skeletal development including, regulation of hormone levels (GO:0010817), hormone metabolic process (GO:0042445), muscle system process (GO:0003012), cellular hormone metabolic process (GO:0034754), muscle contraction (GO:0006936), response to estrogen (GO:0043627), response to estradiol (GO:0032355), regulation of transforming growth factor beta receptor signalling pathway (GO:0017015), skeletal system morphogenesis (GO:0048705), embryonic skeletal system morphogenesis (GO:0048704), collagen fibril organization (GO:0030199), regulation of hormone biosynthetic process (GO:0046885), bone trabecula formation (GO:0060346) and negative regulation of hormone biosynthetic process (GO:0032353). These gene ontologies showed hormone and estrogen related genes with ALDH1A2, ANXA1, COL1A1, ESR1, KRT19, NPY1R, PDGFRA, IFI27, and IGF2 under response of estrogen and estradiol.

**Table 3-8: Functions Enrichment Analysis Result of Mg-63 sequences**

<b>Term</b>	<b>P-value</b>	<b>No. of genes</b>	<b>Members of the panel of 262 estrogen dependent genes</b>
<b>Regulation of hormone levels (GO:0010817)</b>	4.67E-05	12	PDGFRA,ANXA1,UGT1A1,ALDH1A2,CTSZ,LTPB4,CACNA1A,UGT1A3,UGT1A9,ESR1,UGT1A8,UGT1A7
<b>Hormone metabolic process (GO:0042445)</b>	4.16E-05	10	PDGFRA,UGT1A1,ALDH1A2,CTSZ,CACNA1A,UGT1A3,UGT1A9,ESR1,UGT1A8,UGT1A7
<b>Muscle system process (GO:0003012)</b>	0.001089	10	GSTM2,MEF2C,VIPR1,DTNA,CCDC78,CALD1,PDE4D,TPM2,KCNMA1,MYOM2
<b>Cellular hormone metabolic process (GO:0034754)</b>	3.27E-05	8	PDGFRA,UGT1A1,ALDH1A2,UGT1A3,UGT1A9,UGT1A8,ESR1,UGT1A7
<b>Muscle contraction (GO:0006936)</b>	0.003993	8	VIPR1,DTNA,CCDC78,CALD1,PDE4D,TPM2,KCNMA1,MYOM2
<b>Response to estrogen (GO:0043627)</b>	0.0199	7	ALDH1A2,ANXA1,COL1A1,ESR1,KRT19,NPY1R,PDGFRA
<b>Response to estradiol (GO:0032355)</b>	0.001923	6	COL1A1,ANXA1,IFI27,ALDH1A2,IGF2,ESR1
<b>Regulation of transforming growth factor beta receptor signaling pathway (GO:0017015)</b>	0.002233	6	DAB2,ZEB1,TGFB1,LTPB4,LDLRAD4,FBN1
<b>Skeletal system morphogenesis (GO:0048705)</b>	0.002581	6	PDGFRA,ZEB1,HOXB4,HOXB3,HOXB6,FGFR1
<b>Embryonic skeletal system morphogenesis (GO:0048704)</b>	0.004034	5	PDGFRA,ZEB1,HOXB4,HOXB3,HOXB6
<b>Collagen fibril organization (GO:0030199)</b>	0.00191	4	COL1A1,COL3A1,COL1A2,COL14A1
<b>regulation of hormone biosynthetic process (GO:0046885)</b>	0.002755	3	IGF2,CACNA1A,DKK3
<b>Bone trabecula formation (GO:0060346)</b>	0.004443	2	COL1A1, MMP2
<b>negative regulation of hormone biosynthetic process (GO:0032353)</b>	0.004443	2	CACNA1A,DKK3

Enriched functions of up and down regulated genes are listed and separated into GO terms of biological process category. Bold genes are that among the list of 55 Mg-63 genes.

### 3.3 Discussion

The aim of this chapter was to identify differentially-expressed estradiol-responsive genes in cultured osteosarcoma cell lines (Mg-63, TE-85 and Saos-2) by RNA Sequencing. This was done in order to identify differential gene changes relevant to the development of osteoporosis in postmenopausal women who are more susceptible to this disease when estrogen is reduced.

The results showed that both ER  $\alpha$  and ER  $\beta$  mRNA were detected in all three osteosarcoma cells, with protein only detected in Mg-63 cells. The results of the present experiments are similar to that the previously reported (Lambertini et al., 2003, Solakidi et al., 2005, Kurosawa et al., 2002). However, ER  $\alpha$  mRNA and protein showed much higher level in MCF-7 than that in any of the osteosarcoma cells, which is not unexpected, due to very high levels of ER  $\alpha$  in the MCF-7 cells (Lewandowski et al., 2005, Grober et al., 2011, Jonsson et al., 2014). The results also demonstrated that upon treatment with  $\beta$ -estradiol, the proliferation rates of the cultured cells were increased in Mg-63, TE-85, Saos-2 and MCF-7 compared to the untreated cells. This result is similar to other previous reports suggesting that estradiol can stimulate the growth of osteosarcoma cells (Lajeunesse, 1994), and TE-85 cells (Ikegami et al., 1994). Importantly, the proliferative capabilities of Mg-63 cells has been shown to be positively influenced by circulating  $\beta$ -estradiol levels in a dose-dependent fashion (Dohi et al., 2008). Collectively, it is known that estrogen has a bone-protective effect, mediated via the estrogen receptors  $\alpha$  and/or  $\beta$  (Khalid and Krum, 2016). Since ER  $\alpha$  proteins were present in the Mg-63 cells, with a very low amount in TE-85 and Saos-2 cells, this suggests that the reason for small number of differentially expressed genes in TE-85 and Saos-2 treated cells was due to the low level of ER  $\alpha$  proteins in both cell lines. It has

been reported that  $\beta$ -estradiol increased osteoblast proliferation through negative regulation of Sclerostin (SOST) expression in an indirect manner through interaction with BMP2 signalling and that this regulation involves the Wnt/ER $\alpha$  and  $\beta$ -catenin pathways (Kim et al., 2015b, Galea et al., 2013a). Thus, because of detecting ER  $\beta$  mRNA and protein in all three osteosarcoma cells, these results suggest that ER  $\beta$  might be responsible in the proliferation process of osteoblast cells rather than gene expression which is mediated by ER $\alpha$ , since also RNA-Seq data, in this study, did not showed that ER  $\beta$  was changed in treated osteosarcoma and MCF-7 cells with estradiol compared to the non-treated control.

The alignment of RNA-seq reads to the human reference genome showed that 88.19 % of the total reads were identical to the human reference genome, a value which falls within the accepted range for differential gene expression analysis (Trapnell et al., 2012, Conesa et al., 2016). Remarkably, overall expression of 262 genes in the Mg-63 cells and 279 genes in the MCF-7 cell line were altered. This may correspond broadly with the ER  $\alpha$  receptor status rather than the ER  $\beta$  status of both cells, due to the low level of ER  $\beta$  in the MCF-7 cells. Out of 262 genes altered in the Mg-63 cells, 181 genes were also significantly alerted in the estrogen-treated MCF-7 cells. These common genes were related to regulation of estrogen levels and hormone metabolic process. Therefore, it is suggested that these 181 genes were responsive to  $\beta$ -estradiol and the remaining 81 genes might be osteosarcoma-specific estradiol responsive mRNAs in which both could be involved in the bone turnover pathway.

In the present experiments, IFI16, Interferon Gamma Inducible Protein 16, showed among the highest upregulation, with a change 9.4-fold upon estrogen treatment. IFI16 has an important role in autoimmune disease, in which a high level of IFI16 is



responsible for inducing proinflammatory molecules that lead to apoptosis in endothelial cells (Caposio et al., 2007, Gugliesi et al., 2010). Also, transfection of IFI16 into Saos-2 cells inhibited significantly cell growth and the authors suggested that a certain level of IFI16 is important for organizing the proliferation of bone and cartilage tumor cells and may be used as an antitumor agent (Zhang et al., 2007). The expression of MMP2 was also increased in estrogen-treated Mg-63 cells compared to non-treated control cells by 9.7-fold, as it plays a role in the breakdown of extracellular matrix (ECM) which is important for embryonic development, morphogenesis, and tissue remodeling (Tallant et al., 2010, Nagase and Woessner, 1999). Also it is involved in the degradation of ECM during embryonic bone formation (Sasano et al., 2002, Johansson et al., 1997, Mosig et al., 2007), in which silencing of MMP2 led to an increase in the osteopontin (OPN) and bone sialoprotein (BSP) expression (Mosig and Martignetti, 2013).

The RNA-Seq data showed an increase in the level of TGF $\beta$ I (Transforming Growth Factor Beta Induced) by 6.5 fold due to  $\beta$ -estradiol treatment. This gene is an important regulator of osteoblast differentiation during endochondral ossification (Thorp et al., 1992). The regulation occurs by activating osteoblast recruitment, differentiation, and function and inhibiting terminal osteoblast differentiation, bone matrix synthesis, and osteocyte apoptosis (Tang and Alliston, 2013). TGF $\beta$ I protein binds to type I, II and IV collagens, which play a role in cell-collagen interactions (Hashimoto et al., 1997). Also, TGFBI inhibits the formation of osteoclast precursor cells and bone resorption (Lee et al., 2015). The expression of Annexin A1 (ANXA1) was also shown to be increased by a 6-fold change in  $\beta$ -estradiol -treated Mg-63 cells. it was found to be involved in the regulation of hormone levels and response to estrogen. A previous report suggested that ANXA1 was a tumour suppressor gene since its expression increased

when MCF-7 exposure to estrogen (Ang et al., 2009). ANXA1 can activate NF- $\kappa$ B in breast cancer cells mediated by IKK kinase complex suggesting that modulating ANXA1 levels has therapeutic potential to suppress breast cancer metastasis (Bist et al., 2011). This suggests that suppression of ANXA1 might decrease the activity of NF- $\kappa$ B in bone cells result in inhibiting the osteoclastogenesis process. However, the role of ANXA1 on bone cells is not well understood.

$\beta$ -estradiol increases the expression of VIM, Vimentin, in Mg-63 by 8.8-fold compared to control. VIM is type III intermediate filament (IF) protein which makes up, with microtubules and actin microfilaments, the dynamic cytoskeleton that regulates cell shape, and growth (Olson and Capetanaki, 1989, Capetanaki et al., 1989), enables intracellular transport, and supports cell division (Herrmann and Aebi, 2004). VIM inhibits endogenous osteocalcin transcription and osteoblast differentiation and inhibits the expression of osteoblast marker genes. TGF- $\beta$  increases VIM expression at the post-transcriptional level in osteoblast cells resulting in inhibiting the osteoblast differentiation (Lian et al., 2009, Lian et al., 2012b). The expression of TMEM64, Transmembrane Protein 64, is decreased by -5-fold change in Mg-63 upon estrogen treatment. TMEM64 has expression levels inversely related to those of miR-23a/b in bone marrow mesenchymal stem cells (BMSCs), which is an osteoblast precursor, from aged compared with young mice and humans (Guo et al., 2016) suggesting that TMEM64 is the major target of miR-23a/b during BMSC Differentiation (Guo et al., 2016) suggesting that TMEM64 may play a role in osteoblast maturation. Cathepsin Z (CTSZ) was upregulated by 6.5 fold- in  $\beta$ -estradiol-treated Mg-63 compared to untreated control as well as TRAM2, Translocation associated membrane protein 2, which increased by 5.9-fold. The CTSZ was also found to be involved in the regulation

of hormone levels and hormone metabolic process by the GO analysis. Liu et al (2014) reported that CTSZ and TRAM2 displayed estradiol dependent induction in human fetal osteoblasts transfected with estrogen receptor  $\alpha$ , based on case-cohort genome-wide association study (GWAS). Furthermore, suppression of these genes in human fetal osteoblasts (hFOB)-ER $\alpha$  -containing cell line decreased the expression of other genes in pathways associated with osteoporosis risk and/or bone biology such as RANK-RANKL-OPG (Liu et al., 2014a). RUNX2 increase the osteoblast differentiation and function by promoting the transcription of type I collagen, produced by osteoblast, and also TRAM2, necessary for effective post-translational processing of this macromolecule (Pregizer et al., 2007). Thus, estrogen responsive genes identified in the present study have been shown to be involved in bone remodelling.

In conclusion, all three-osteosarcoma cell lines responded to estradiol by increased growth rate compared to non-treated control, this was probably due to an effect through ER  $\beta$ . RNA seq result showed that the human osteosarcoma cell line, Mg-63 cells, was responsive to estrogen by altering the level of mRNAs and that many genes are affected by the hormonal treatment. Some of these genes were shown to be involved in bone formation. In the next chapter, RT-qPCR will be used, firstly to confirm the differential expression of the RNA-seq-detected genes, and their miRNAs regulators in the three cultured osteosarcoma cell lines and secondly, to find out whether they are relevant to osteoporosis using osteoclast precursors from clinical blood samples.

## **Chapter 4: Identification of Differentially- Expressed mRNAs/miRNAs in Post- Menopausal Osteoporosis**

## 4.1. Introduction

Postmenopausal osteoporosis is the most common type of osteoporosis. The deficiency of ovarian production of estrogen after the menopause is the major cause of rapid bone loss in women (Gallagher and Tella, 2014). In Chapter 3, RNA-sequencing identified several genes that were differentially expressed between the cultured osteosarcoma cell line Mg-63 treated with  $\beta$ -estradiol and non-treated control. Some of these genes found to be estrogen responsive or involved in bone turnover based on gene ontology analysis. Some of the identified genes were known to be involved in the regulation of bone remodelling, for example, DKK3, XAF1, MMP2, COL1A2, IFI44L, IFI16, VIM, MX1, PARP10, TGFBI, CTSZ, COL1A1, TRAM2, TMEM64, KRT8, CELSR2 and ESR1. Other genes were involved in estrogen regulation, such as BLID, ANXA1, CALD1, CTSZ, TRAM2, EMP1, TFAP2C, GREB1, and ESR1.

Several studies indicate the importance of miRNAs in monitoring bone homeostasis and metabolism (Lian et al., 2012a, Eguchi et al., 2013, Laxman et al., 2015, Seeliger et al., 2014, Seeliger et al., 2016, Weilner et al., 2015). However, most of these studies were carried out using cultured cells or animal model systems (van Wijnen et al., 2013) with the exception of few studies in clinical samples from patients who have osteoporosis fractures in small pilot experiments. Our group identified circulating of 6 miRNAs in osteoporosis compare to non-osteoporotic patients (not published yet). However, it is not clear that these identified miRNAs were responsive to  $\beta$ -estradiol or not and can be differentiated between non-osteoporotic and postmenopausal osteoporosis women.

It is well-known that peripheral blood mononuclear cells (PBMCs) participate in osteoclastogenesis by acting as osteoclast precursors (Fujikawa et al., 1996, Matayoshi et al., 1996), migrating to bone through the peripheral circulation (Parfitt et al., 1996, Parfitt). Then, they differentiate and fuse into immature multinuclear osteoclasts, which subsequently become activated to be mature osteoclasts at sites of bone resorption (Roodman, 2006). Although it is difficult to collect bone samples *in vivo* from humans for experimentation, PBMCs can be easily collected in large numbers from a blood sample. Also, changes in estrogen receptors mRNA in the osteoblast have been reported during osteoclast differentiation (Aguirre et al., 2007, Damien et al., 1998). Therefore, identified differentially-expressed mRNA/miRNA from RNA-seq and MicroRNA-PCRArray data were further analysed by RT-qPCR in clinical samples from osteoporosis patients to assess potential diagnostic values for osteoporosis.

## 4.1 Aim

The aims of this chapter are to investigate the panel of differentially-expressed mRNAs from the Mg-63 cell line, identified by RNA sequencing from Chapter 3, and their miRNAs regulators, and their expression in PBMCs of participants was analysed by RT-qPCR. The relationship between mRNAs expression change and BMD was also examined to investigate their combined association with osteoporosis, and to find out whether these mRNAs could be used as diagnostic and therapeutic biomarkers for osteoporosis in the future.

## 4.2. Result

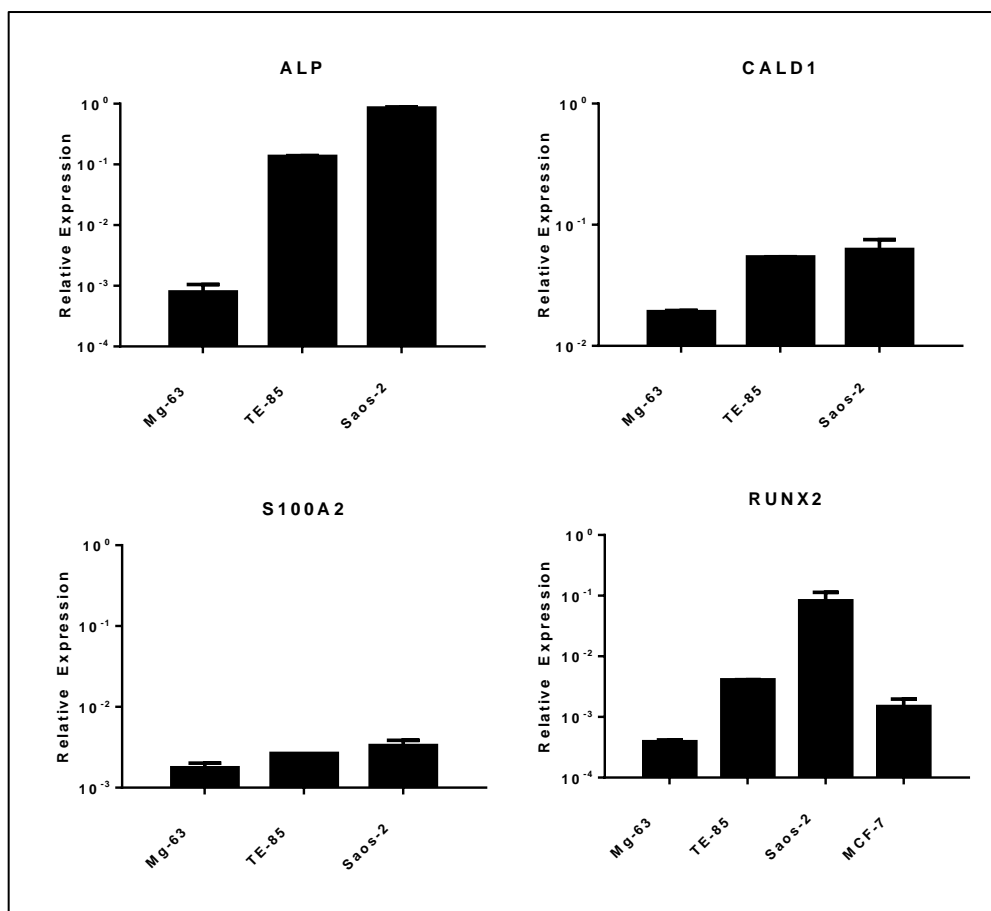
### 4.2.1 Identification of Differentially-Expressed mRNAs in Osteosarcoma Cell Lines

The purpose of this part is to check the identified differentially-expressed genes from RNA-sequencing (Chapter 3) for expression by the three osteosarcoma cell lines. The three cultured osteosarcoma cell lines, Mg-63, TE-85 and Saos-2, represent low, moderate and high degree of differentiation, respectively (Pacheco-Pantoja et al., 2011). The 55 mRNAs, shown in Table 3-5, were selected and their relative expression levels among the cultured cells lines and control MCF-7 cells were determined using RT-qPCR by the  $2^{-\Delta C_t}$  method (Schmittgen and Livak, 2008).

Among the 55-genes, the mRNAs for 10 of the genes were not detected in any of the osteosarcoma cells by the RT-qPCR. These include CREB5, LSP1, CELSR2, FXYP3, GFRA1, GREB1, LLGL2, NPY1R, MLPH, and KRT19. Thus, they were excluded from any further study. However, 45 genes were found to be expressed at different levels in the cultured osteosarcoma cells.

The expressions of four mRNAs, ALP (Alkaline Phosphatase), CALD1 (Caldesmon 1), S100A2 (S100 calcium binding protein A2) and RUNX2 (Runt related transcription factor 2) were lowest in Mg-63, and highest in Saos-2, while the expression of these genes in TE -85 was between that in Mg-63 and Saos-2 (**Figure 4-1**). The expression levels of ALP mRNA in Saos-2 and TE-85 were 1068 and 170-fold higher compared to its levels in Mg-63 respectively. The expression levels of CALD1 mRNA in Saos-2 and TE-85 were 3.3 and 2.8-fold higher than that in Mg-63, respectively. The expression levels of S100A2 mRNA in Saos-2 and TE-85 were 1.9 and 1.5-fold higher

than that in Mg-63. RUNX2 was also highly expressed in Saos-2 and TE-85 with levels of 150 and 7 respectively than that in Mg-63. The results show increasing levels of these mRNAs with increasing maturation of cells, the lowest maturation cells, Mg-63 contains lowest levels of these mRNAs and highest maturation cells Saos-2 contains highest levels of these mRNAs. The results suggest that these mRNAs might be linked with the degree of maturation of the osteosarcoma cells.



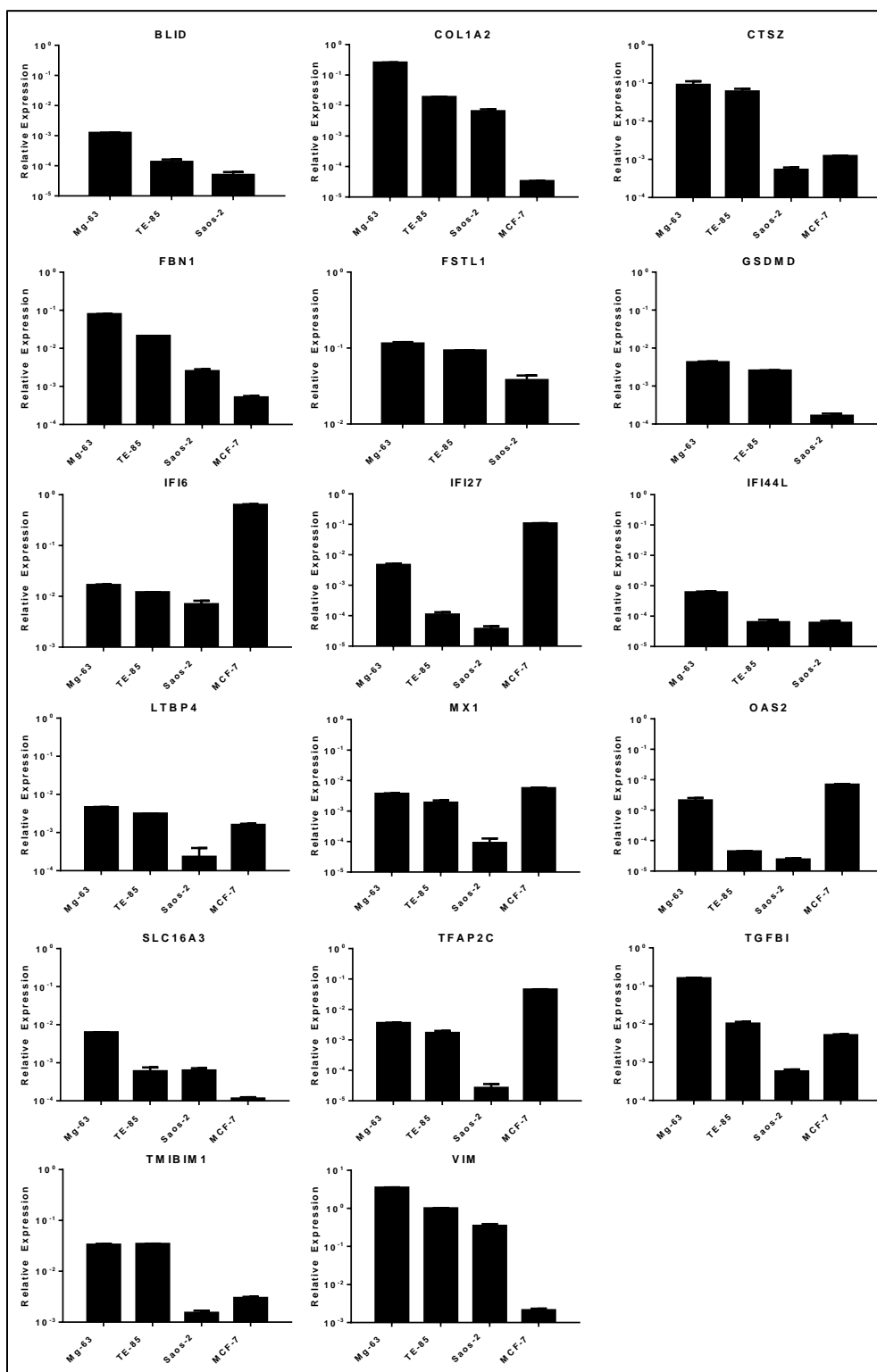
**Figure 4-1: Levels of mRNAs for differentially expressed genes increase in osteosarcoma cell lines**

Relative expression of mRNA was determined by the relative Ct method normalized to GAPDH (Data presented as Log 10 of  $2^{-\Delta Ct}$ ). The figure represents the expression of ALP, (B) for CALD1, (C) for S100A2, and (D) for RUNX2. Each bar represents the mean and  $\pm$ SD (n=3).



On the other hand, the expression levels of 17 mRNAs showed a decreased in their expression from Mg-63 through TE-85 to Saos-2 (**Figure 4-2**). These mRNAs are, BLID (BH3-like motif containing, cell death inducer), COL1A2 (Collagen type I alpha 2 chain), CTSZ (Cathepsin Z), FBN1 (Fibrillin 1), FSTL1 (Follistatin like 1), GSDMD (Gasdermin D), IFI6 (Interferon alpha inducible protein 6), IFI27 (Interferon alpha inducible protein 27), IFI44L (Interferon induced protein 44 like), LTBP4 (Latent transforming growth factor beta binding protein 4), MX1 (MX dynamin like GTPase 1), OAS2 (2'-5'-oligoadenylate synthetase 2), SLC16A3 (Solute carrier family 16 (monocarboxylic acid transporters), member 3), TFAP2C (Transcription factor AP-2 gamma), TGF $\beta$ 1 (Transforming growth factor beta induced), TMIBIM1 (Transmembrane BAX inhibitor motif containing 1), and VIM (Vimentin).

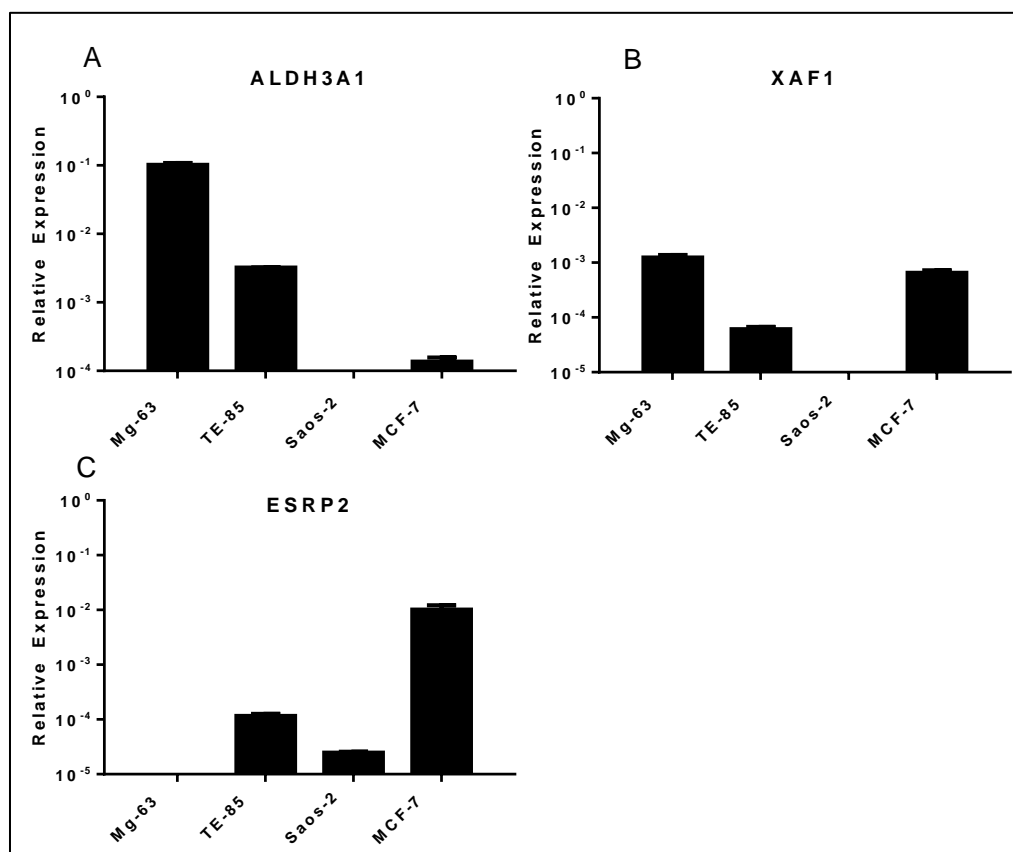
The expression levels of BLID and COL1A2 mRNA in Mg-63 were 25 and 39-fold higher compared to its levels in Saos-2 respectively, and 3-fold higher for both mRNAs in TE-85 that that in Saos-2. The expression levels of CTSZ mRNA in Mg-63 and TE-85 were 168 and 113-fold higher compared to its levels in Saos-2 respectively. The expression levels of FBN1 mRNA in Mg-63 and TE-85 were 31 and 8-fold higher than that in Saos-2. The expression levels of GSDMD mRNA in Mg-63 and TE-85 were 26 and 16-fold higher than that in Saos-2. IFI27 was highly expressed in Mg-63 and TE-85 with levels of 126 and 3 respectively than that in Saos-2. The expression levels of TGFBI mRNA in Mg-63 and TE-85 were 277 and 18-fold higher than that in Saos-2. The expression levels of VIM mRNA in Mg-63 and TE-85 were 10 and 3-fold higher than that in Saos-2. The results showed that the levels of these 17 mRNAs exhibited an inverse relationship with increasing maturity of the osteosarcoma cells.



**Figure 4-2: Inversely relationship of mRNAs expression and the maturity of osteosarcoma cell lines**

Relative expression of mRNA was determined by the relative Ct method normalized to GAPDH (Data presented as  $\text{Log } 10 \text{ of } 2^{-\Delta C_t}$ ). these genes showed a decrease in their expression with increased level of maturation. Each bar represents the mean  $\pm$ SD (n=3).

The expression levels of 2 mRNAs, ALDH1A1 and XAF1 were higher in Mg-63 compared to that in TE-85 by 32 and 20-fold respectively, but they were not detected in Saos-2 (**Figure 4-3A,B**). The expression of ESRP2 was higher in TE-85 by 5-fold compare to that in Saos-2. However, ESRP2 was not expressed in Mg-63, **Figure 4-3C**.



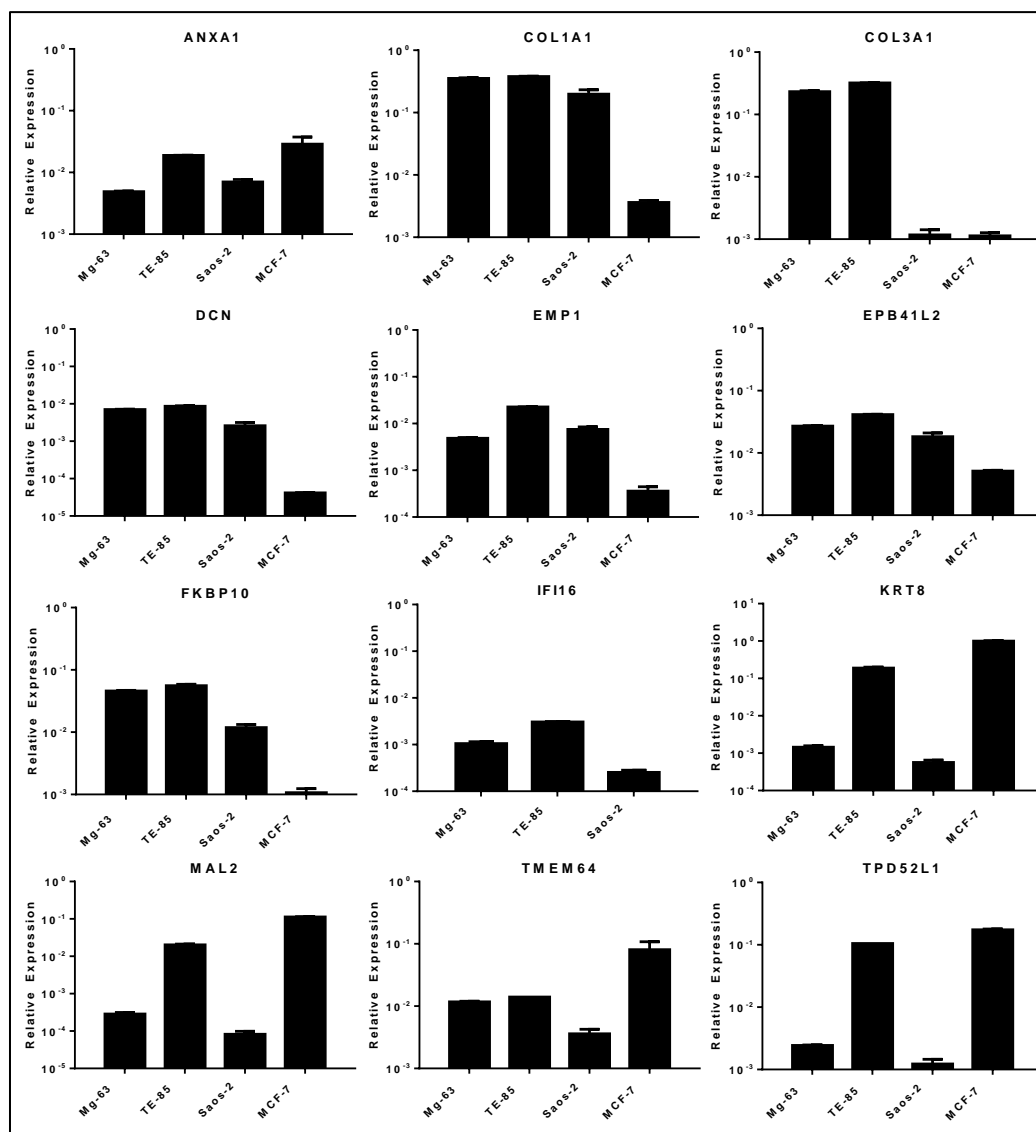
**Figure 4-3: RT-qPCR of differentially expressed mRNAs in osteosarcoma cell line**  
Relative expression of mRNA was determined by the relative Ct method normalized to GAPDH (Data presented as Log 10 of  $2^{-\Delta Ct}$ ). ALDH3A1 and XAF1 were not expressed in Saos-2, ESRP2 was not expressed in Mg-3. Each bar represents the mean  $\pm$ SD (n=3).

The expression levels of 12 mRNAs, ANXA1(Annexin A1), COL1A1 (Collagen type I alpha 1 chain), COL3A1 (Collagen type III alpha 1 chain), DCN (Decorin), EMP1 (Epithelial membrane protein 1), EPB41L2 (Erythrocyte membrane protein band 4.1 like 2), FKBP10 (FK506 binding protein 10), IFI16 (Gamma-interferon-inducible protein 16) KRT8 (Keratin 8), MAL2 (Mal, T-cell differentiation protein 2), TMEM64

(Transmembrane protein 64), TPD52L1 (Tumor protein D52 like 1), in Mg-63 and Saos-2 were lower than in TE-85, (**Figure 4-4**).

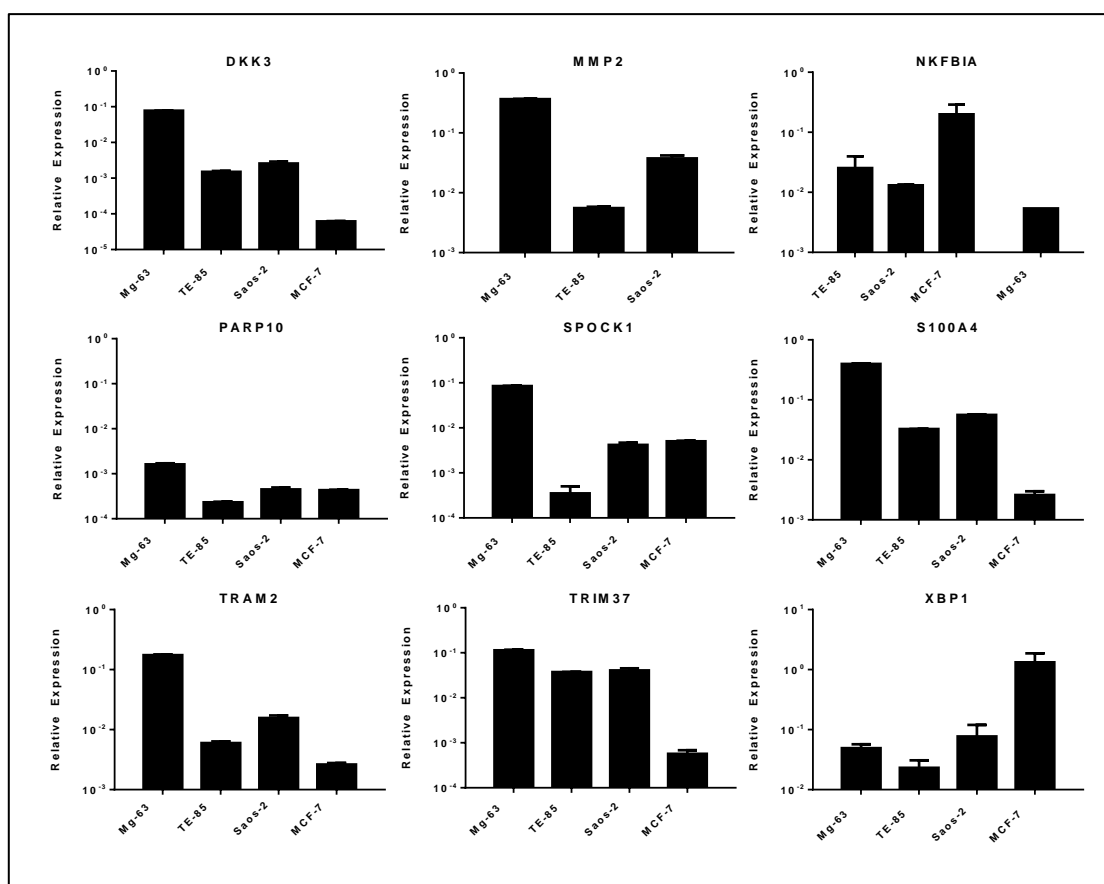
On the other hand, the expression of DKK3 (Dickkopf-related protein 3), MMP2 (Matrix metalloproteinase 2), NFKBIA (NFKB inhibitor alpha), PARP10 (Poly(ADP-ribose) polymerase family member 10), SPOCK1 (SPARC/osteonectin, cwcx and kazal like domains proteoglycan 1), S100A4 (S100 Calcium Binding Protein A4), TRAM2 (Translocation associated membrane protein 2), TRIM37 (Tripartite motif containing 37) XBP1 (X-box binding protein 1) were lower in TE-85 compare to that in Mg-63 and Saos-2, **Figure 4-5**). Thus, different levels of these mRNAs in these different levels of maturation, indicating that changes in levels of these mRNAs might be associated with the development of osteosarcoma cells.

Nine mRNAs, ALP, CALD1, BLID, FSTL1, GSDMD, IFI16, IFI44L, S100A2 and SLC16A3 were detected only in the three osteosarcoma cell lines, but not detected in MCF-7 cells. The results suggest that these genes might be bone related estrogen responsive genes. Overall, the mRNAs that showed different levels of expression between the cell lines, were tested for their response to estrogen.



**Figure 4-4: Lower expression of mRNAs in Mg-63 and Saos-2 than in TE-85 by RT-qPCR**

Relative expression of mRNA was determined by the relative Ct method normalized to GAPDH (Data presented as Log 10 of  $2^{-\Delta Ct}$ ). the expression of these genes showed to be higher in TE-85 and lower in Mg-63 and Saos-2. Each bar represents the mean and  $\pm$ SD (n=3).



**Figure 4-5: Lower expression of mRNAs in TE-85 than in Mg-63 and Saos-2 by RT-qPCR**

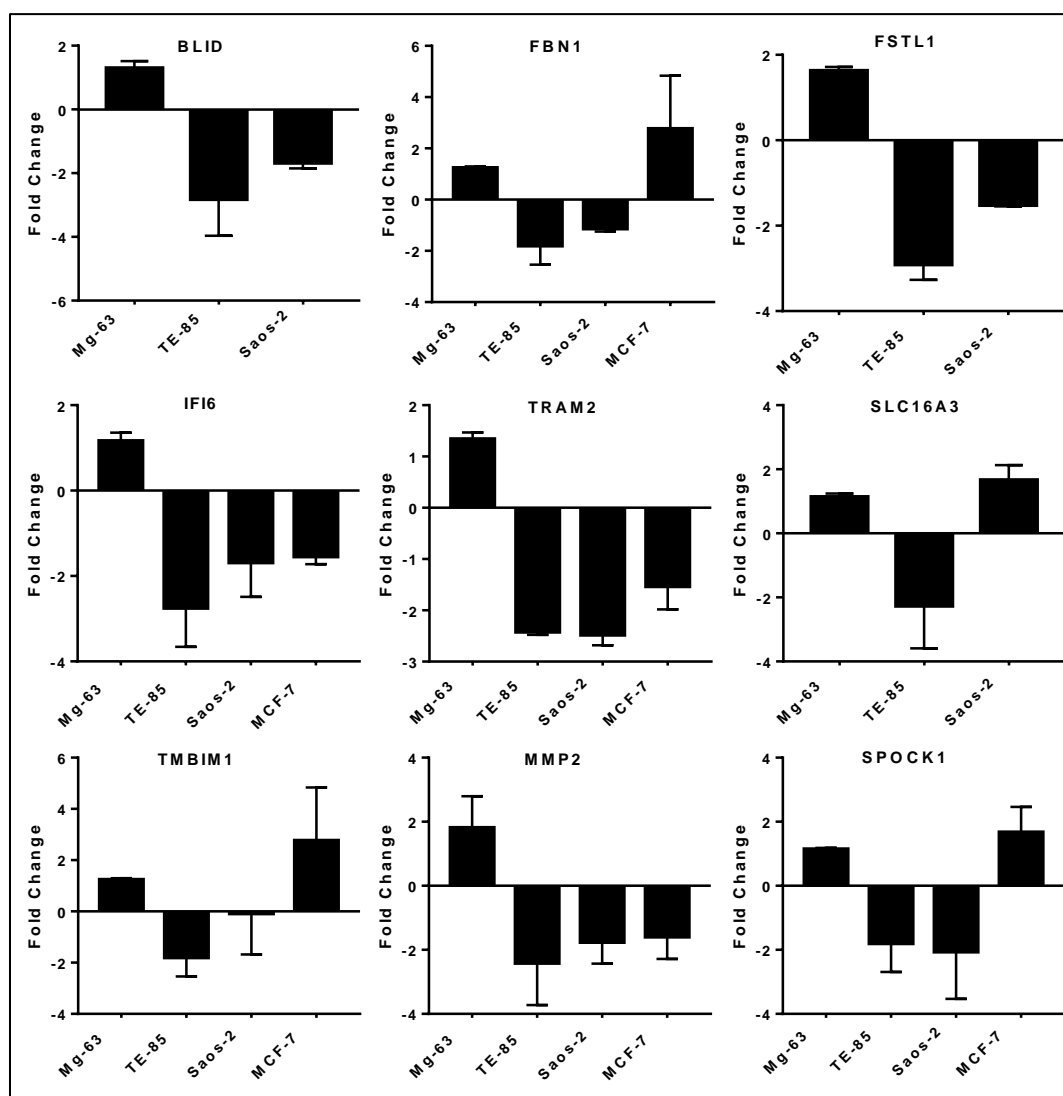
Relative expression of mRNA was determined by the relative Ct method normalized to GAPDH (Data presented as Log 10 of  $2^{-\Delta Ct}$ ). The expression of these genes showed to be lowest in TE-85 than that in Mg-63 and Saos-2. Each bar represents the mean and  $\pm$ SD (n=3).

## 4.2.2 Identification of Differentially-Expressed Estrogen Responsive Genes in Osteosarcoma Cells

The effect of estrogen on the expression of the 45 mRNAs expressed in the osteosarcoma cells (Section 4.2.1) was tested in osteosarcoma cells using RT-qPCR. Nine mRNAs, BLID, FBN1, FSTL1, IFI6, TRAM2, SLC16A3, SPOCK1, TMBIM1, and MMP2 exhibited > three-fold lower expression in the three osteosarcoma cell lines, Mg-63, TE85 and Saos-2 upon  $\beta$ -estradiol treatment compared to the corresponding untreated control cells (**Figure 4-6** and **Table 4-1**).  $\beta$ -estradiol decreased by >2-fold the expression of ALP, COL1A2, COL3A1, EMP1, KRT8, LTBP4, MAL2, TRIM37 and TFAP2C mRNAs in all osteosarcoma cells compared to non-treated control (**Figure 4-7**). The levels of 8 mRNAs, ANXA1, CTSZ, IFI16, GSDMD, S100A2, TGFBI, VIM and XFA1 mRNAs were highest in Mg-63 cells but lower in TE-85 and Saos-2 cells by >2 fold upon estrogen treatment compared to the corresponding non-treated control cells (**Figure 4-8**). The expression levels of DCN and FKBP10 mRNAs, were decreased in TE-85 and Saos-2 compared to non-treated control, but not changed in Mg-63 (**Figure 4-9**). The RT-qPCR data revealed  $\beta$ -estradiol decreased the expression of TMEM64 and ESR1 in Mg-63 and TE-85 compared to non-treated controls, but these mRNAs were increased in Saos-2 cells (**Figure 4-9**). RT-qPCR data showed that  $\beta$ -estradiol decreased the level of 15 mRNAs, ALDH3A1, CALD1, COL1A1, DKK3, ESRP2, EPB41L2, IFI27, IFI44L, NFKBIA, MX1, OAS2, PARP10, RUNX2, S100A4, and XBP1, in TE-85 upon estrogen treatment, but these mRNAs were not changed in Mg-63 and Saos-2 (**Figure 4-10**).

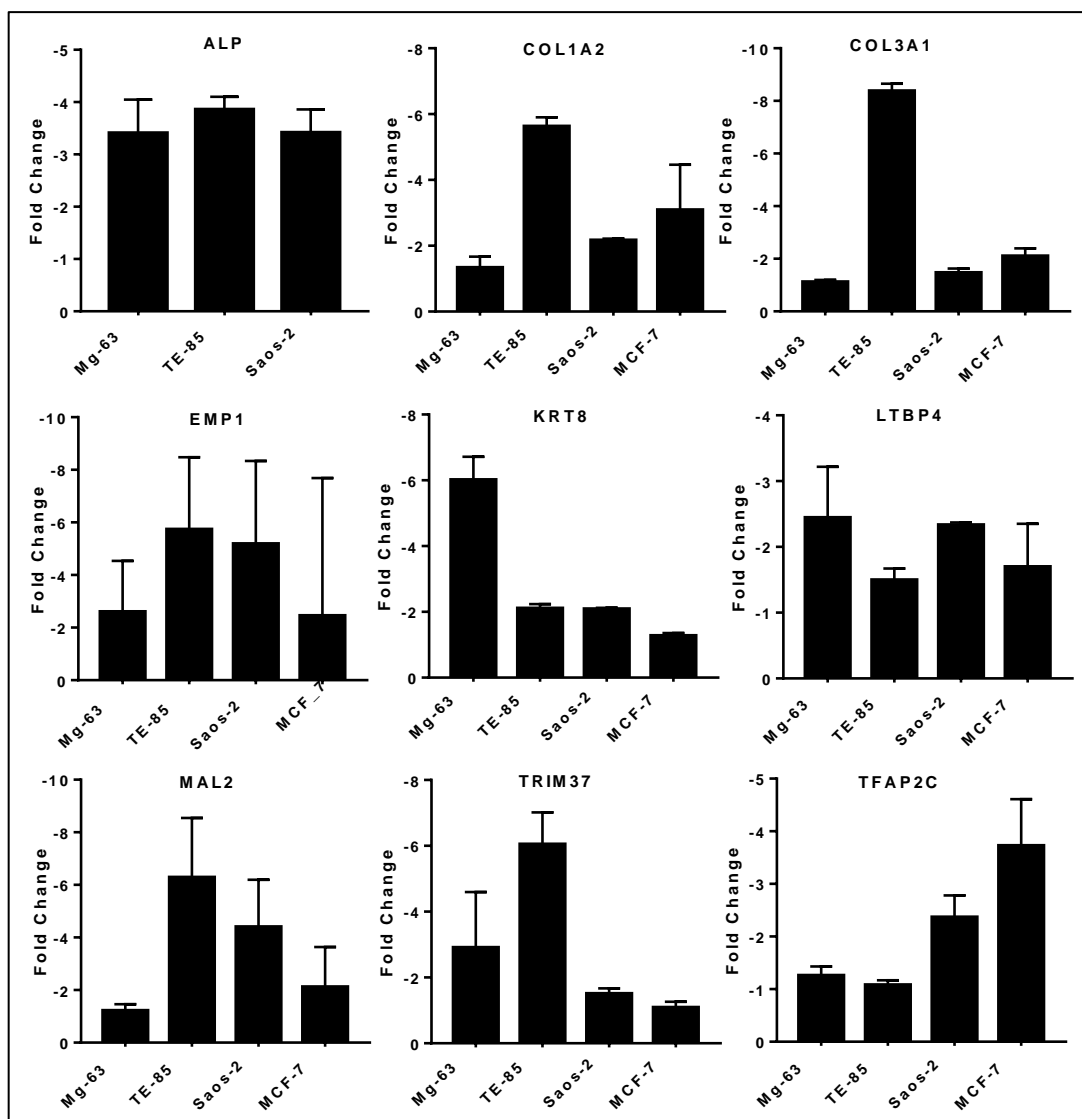
Generally, although these genes showed a different level of expression in the osteosarcoma cell lines with different stages of maturation in a normal condition, the RT-qPCR data suggest that these genes were negatively responsive to  $\beta$ -estradiol. Therefore, in view of the association of osteoporosis with reduced estrogen levels in patients, it is important to find out if these mRNAs change in clinical samples of post-menopausal osteoporosis (Section 4.2.4). However, first, the miRNAs associated with the expression of these 45 genes were identified.





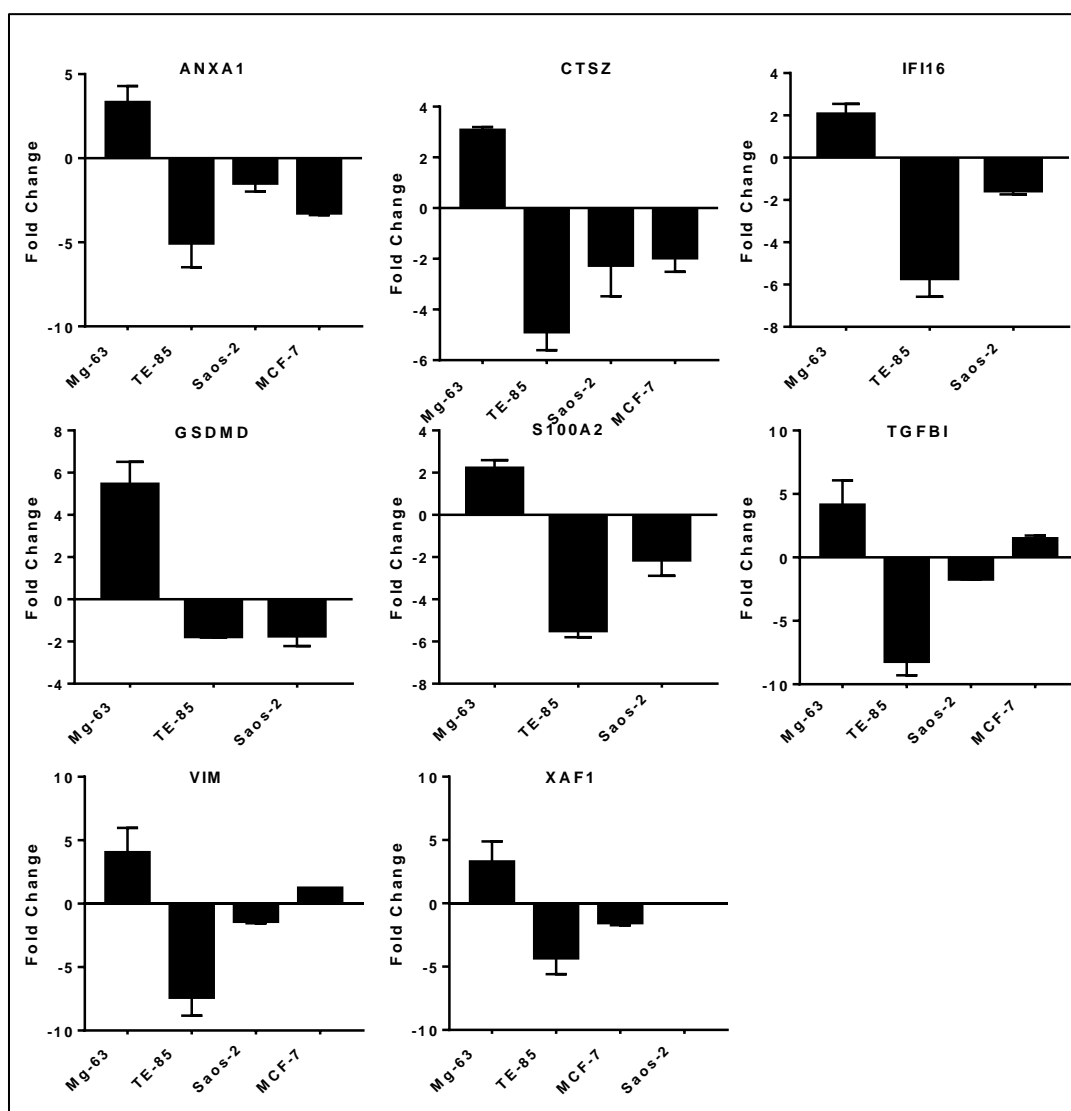
**Figure 4-6: Effect of  $\beta$ -estradiol on mRNA in the three osteosarcoma cell lines by RT-qPCR**

$\leq 3$  Fold Change upon  $\beta$ -estradiol treatment on osteosarcoma cells, fold changes were determined by the relative Ct method normalized to GAPDH ( $2^{-\Delta\Delta C_t}$ ). Each bar represents the mean and  $\pm$ SD (n=3).



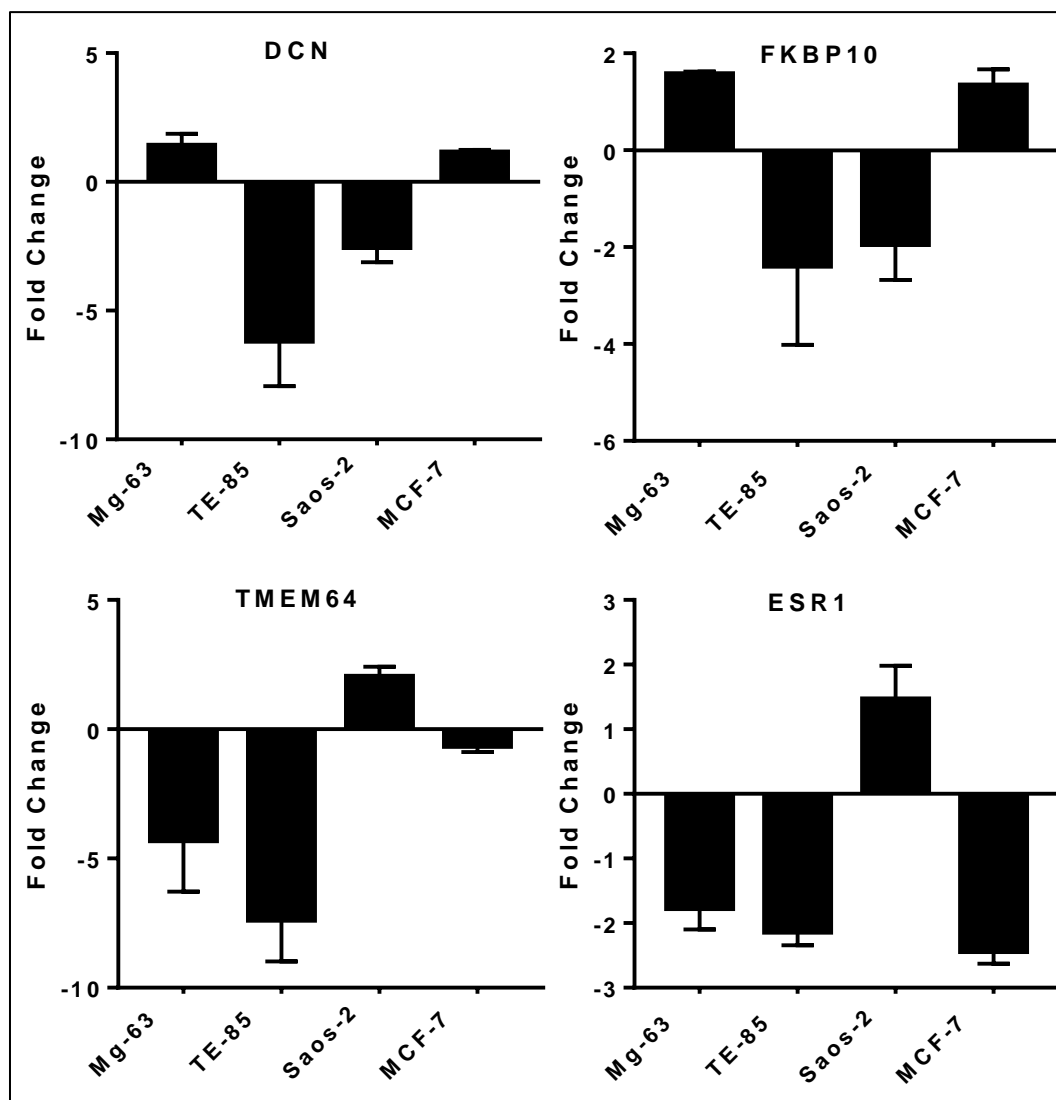
**Figure 4-7: RT-qPCR result of genes that  $\beta$ -estradiol decrease their expression in the all three osteosarcoma cell lines**

Fold Change of genes were determined by the relative Ct method normalized to GAPDH ( $2^{-\Delta\Delta Ct}$ ). Each bar represents the mean and  $\pm$ SD (n=3)..



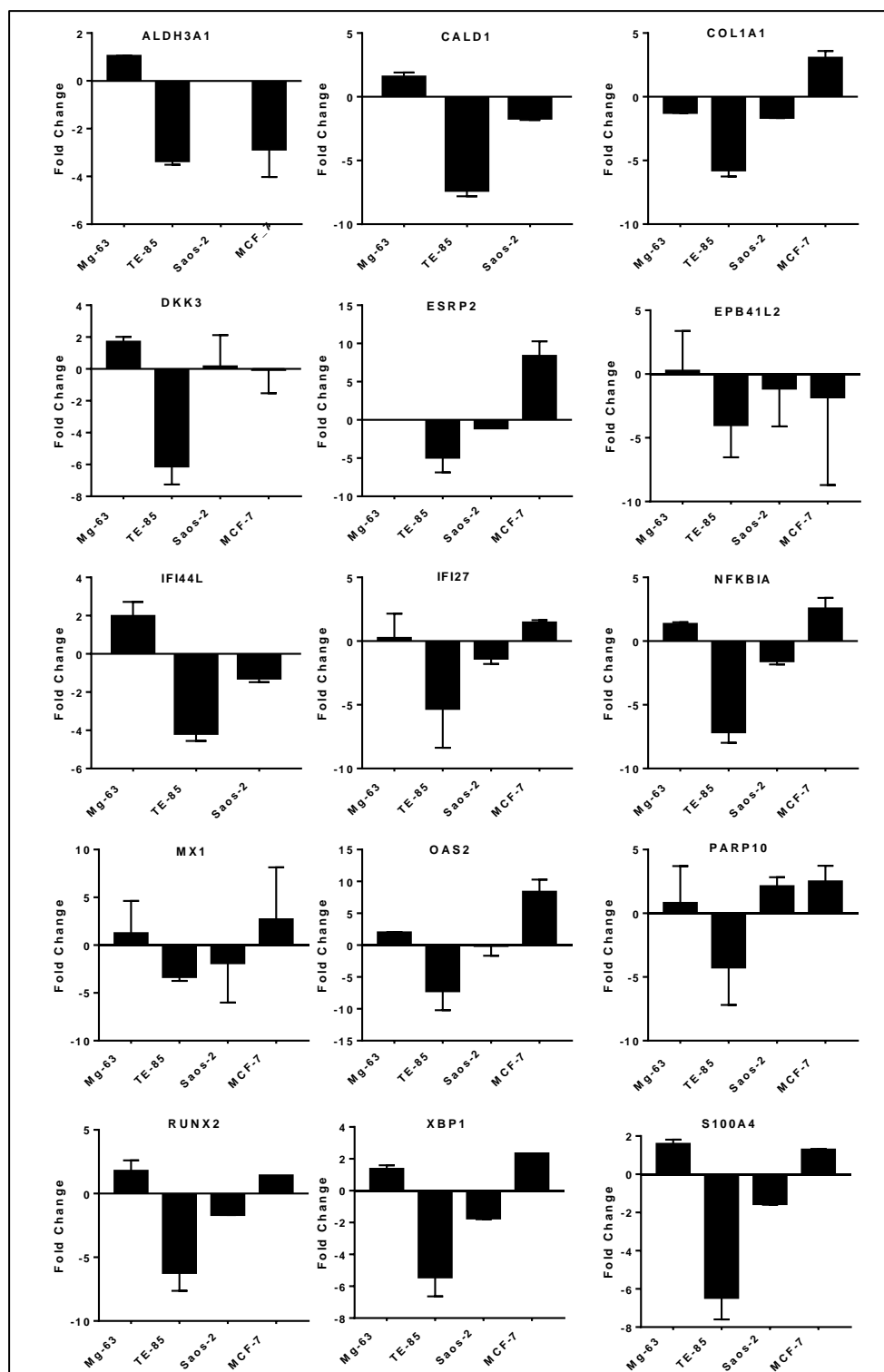
**Figure 4-8: mRNAs that increased in Mg-63 but decreased in TE-85 and Saos-2 upon  $\beta$ -estradiol treatment by RT-qPCR**

Fold Change of genes were determined by the relative Ct method normalized to GAPDH ( $2^{-\Delta\Delta C_t}$ ). Each bar represents the mean and  $\pm$ SD (n=3).



**Figure 4-9: The effect of  $\beta$ -estradiol on DCN, FKBP10, TMEM64, and ESR1 by RT-qPCR**

$\beta$ -estradiol decrease level of DCN and FKBP10 in TE-85 and Saos-2 but increase in Mg-63. Whereas level of TMEM64 and ESR1 were decreased in Mg-63 and TE-85 but increased in Saos-2. Fold Change of genes were determined by the relative Ct method normalized to GAPDH ( $2^{-\Delta\Delta C_t}$ ). Each bar represents the mean and  $\pm$ SD (n=3).



**Figure 4-10:  $\beta$ -estradiol decreased the level of mRNAs in TE-85 without changed in Mg-63 and Saos-2 compared to non-treated control**

Fold Change of genes were determined by the relative Ct method normalized to GAPDH ( $2^{-\Delta\Delta C_t}$ ). Each bar represents the mean and  $\pm$ SD (n=3).

**Table 4-1: The effect of estrogen on the expression of genes selected based on showing different level in the normal condition.**

	Gene	Fold change in Mg-63	Fold change in TE-85	Fold change in Saos-2	Fold change in MCF-7
1	ALDH3A1	1.03 ± 0.03	-3.35 ± 0.17	-	-2.86 ± 1.18
2	ALP	-3.41 ± 0.66	-3.86 ± 0.25	-3.42 ± 0.46	-
3	ANXA1	2.64 ± 0.04	-5.04 ± 1.47	-1.47 ± 0.51	-3.25 ± 0.13
4	BLID	1.31 ± 0.22	-2.83 ± 1.15	-1.68 ± 0.17	-
5	CALD1	1.57 ± 0.35	-7.35 ± 0.47	-1.68 ± 0.15	-
6	COL1A1	-1.23 ± 0.05	-5.75 ± 0.51	-1.63 ± 0.05	3.04 ± 0.57
7	COL1A2	-1.33 ± 0.34	-5.63 ± 0.28	-2.17 ± 0.06	-3.08 ± 1.39
8	COL3A1	-1.1 ± 0.08	-8.38 ± 0.29	-1.47 ± 0.18	-0.22 ± 2.97
9	CTS2	3.07 ± 0.15	-4.87 ± 0.74	-2.25 ± 1.24	-1.96 ± 0.56
10	DCN	1.41 ± 0.46	-6.18 ± 1.77	-2.54 ± 0.59	1.15 ± 0.1
11	DKK3	1.69 ± 0.33	-6.1 ± 1.16	0.13 ± 2.01	-0.03 ± 1.5
12	ESR1	-2.61 ± 1.95	-5.73 ± 2.76	-5.19 ± 3.17	-2.45 ± 5.24
13	EMP1	0.24 ± 3.17	-3.98 ± 2.56	-1.1 ± 3.02	-1.79 ± 6.93
14	EPB41L2	-2.63 ± 1.96	-2.14 ± 0.22	1.51 ± 0.52	-2.44 ± 0.2
15	ESRP2	-	-4.89 ± 1.98	-1.05 ± 0.01	8.32 ± 1.98
16	FBN1	1.24 ± 0.08	-1.81 ± 0.74	-0.08 ± 1.6	3.26 ± 1.38
17	FKBP10	-0.05 ± 2.23	-2.39 ± 1.63	-1.94 ± 0.75	0.24 ± 1.91
18	FSTL1	1.63 ± 0.1	-2.91 ± 0.37	-1.53 ± 0.03	-
19	GSDMD	5.45 ± 1.07	-1.77 ± 0.07	-1.73 ± 0.5	-
20	IFI16	2.05 ± 0.5	-5.72 ± 0.87	-1.57 ± 0.18	-
21	IFI27	0.23 ± 1.95	-5.29 ± 3.1	-1.35 ± 0.45	1.43 ± 0.22
22	IFI44L	1.97 ± 0.75	-4.17 ± 0.39	-1.29 ± 0.21	-
23	IFI6	-1.17 ± 0.2	-2.75 ± 0.92	-1.69 ± 0.82	1.56 ± 0.18
24	KRT8	-6.01 ± 0.71	-2.12 ± 0.14	-2.09 ± 0.05	-1.28 ± 0.08
25	LTBP4	-2.45 ± 0.78	-1.5 ± 0.19	-2.34 ± 0.05	-1.7 ± 0.66
26	MAL2	-1.22 ± 0.26	-3.28 ± 2.27	-1.28 ± 3.4	-2.12 ± 1.54
27	MMP2	1.82 ± 1	-0.93 ± 3.43	-1.77 ± 0.68	-1.6 ± 0.71
28	MX1	1.22 ± 3.43	-3.3 ± 0.46	-1.85 ± 4.17	2.65 ± 5.5
29	NFKBIA	1.33 ± 0.16	-7.12 ± 0.87	-1.55 ± 0.3	2.53 ± 0.87
30	OAS2	1.93 ± 0.15	-7.16 ± 3.06	-0.06 ± 1.62	8.32 ± 1.98
31	PARP10	0.78 ± 2.93	-4.23 ± 2.98	2.1 ± 0.75	2.47 ± 1.28
32	RUNX2	1.76 ± 0.87	-6.2 ± 1.45	-1.66 ± 0.04	1.39 ± 0
33	S100A2	2.21 ± 0.39	-5.48 ± 0.32	-2.13 ± 0.75	-
34	S100A4	1.58 ± 0.25	-4.96 ± 3.28	-1.55 ± 0.07	-0.26 ± 1.82
35	SLC16A3	0.08 ± 1.62	-0.94 ± 3.22	-0.33 ± 2.35	-
36	SPOCK1	1.15 ± 0.05	-1.81 ± 0.89	-2.06 ± 1.48	1.67 ± 0.8
37	TFAP2C	-1.26 ± 0.17	-1.08 ± 0.1	-2.37 ± 0.42	-3.73 ± 0.89
38	TGFβ1	4.1 ± 1.98	-8.18 ± 1.12	-1.69 ± 0.06	1.46 ± 0.28
39	TMBIM1	1.24 ± 0.08	-1.81 ± 0.74	-0.08 ± 1.6	2.76 ± 2.08

	Gene	Fold change in Mg-63	Fold change in TE-85	Fold change in Saos-2	Fold change in MCF-7
40	<b>TMEM64</b>	$-4.3 \pm 1.98$	$-7.38 \pm 1.62$	$2.03 \pm 0.39$	$1.46 \pm 0.19$
41	<b>TRAM2</b>	$1.29 \pm 0.3$	$-2.43 \pm 0.07$	$-2.48 \pm 0.22$	$-1.54 \pm 0.46$
42	<b>TRIM37</b>	$-2.9 \pm 1.7$	$-6.05 \pm 0.97$	$-1.51 \pm 0.17$	$0.07 \pm 1.93$
43	<b>VIM</b>	$4 \pm 1.98$	$-7.37 \pm 1.47$	$-1.38 \pm 0.17$	$1.22 \pm 0$
44	<b>XAF1</b>	$3.26 \pm 1.64$	$-4.29 \pm 1.31$	$-1.51 \pm 0.22$	-
45	<b>XBP1</b>	$1.35 \pm 0.25$	$-5.43 \pm 1.21$	$-1.73 \pm 0.07$	$2.32 \pm 0$

Fold Changes of differentially-expressed mRNAs of genes in  $\beta$ -estradiol- treated osteosarcoma cell lines which were calculated by the relative Ct method normalized to GAPDH ( $2^{-\Delta\Delta C_t} \pm SD$  (n=3)).

## 4.2.3 Potential Target miRNAs in Osteosarcoma Cells

### 4.2.3.1 Identification of miRNAs for estradiol responsive mRNAs

The potential target miRNAs for the identified 45 estrogen responsive genes (Section 0) were identified bioinformatically. These miRNAs were identified using 4 different algorithms (miRanda, miRDB, miRWalk and Targetscan) within the miRWalk 2.0 database. The results are summarized in **Table 4-2**. The data show that hsa-miR-100 may hit 8 of the estrogen responsive mRNAs, ANXA1, COL1A1, COL1A2, COL3A1, DCN, ESR1, FBN1, and FSTL1. hsa-miR-196 is reported to regulate 11 genes, ANXA1, CALD1, COL1A1, COL1A2, COL3A1, DCN, DKK3, EPB41L2, ESR1, FBN1 and FKBP10. COL1A1, CTSZ, EMP1, FKBP10 and FSTL1 can be controlled by has-miR-1260. Has-miR-1290 might regulate the expression of CALD1, COL3A1, DCN, DKK3, EMP1, ESR1, FBN1, FKBP10 and FSTL1. Has-miR-143 can regulate 8 mRNAs, BLID, CALD1, COL1A2, CTSZ, EMP1, ESR1, FBN1, and FKBP10. Has-miR-145 can regulate 6 mRNAs, BLID, COL1A1, DCN, EMP1, EPB41L2, ESR1. Has-miR-373 can regulate 9 mRNAs CALD1, COL1A1, COL1A2, DCN, DKK3, EMP1, EPB41L2, ESR1, FSTL1. ANXA1, CALD1, COL1A2, DCN, EMP1, EPB41L2, ESR1, FBN1 and FSTL1 can be regulated by has-miR-409. Lastly, FKBP10 might be regulated by has-miR-99a.



**Table 4-2: Potential target miRNAs of estrogen related genes**

<b>Gene</b>	<b>miRNA</b>	<b>miRanda</b>	<b>miRDB</b>	<b>miRWalk</b>	<b>Target scan</b>	<b>SUM</b>
<b>ANXA1</b>	hsa-miR-196a	1	0	1	1	3
<b>ANXA1</b>	hsa-miR-196b	1	0	1	1	3
<b>ANXA1</b>	hsa-miR-100	1	1	0	0	2
<b>ANXA1</b>	hsa-let-7c	1	0	1	0	2
<b>ANXA1</b>	hsa-miR-409	0	0	1	0	1
<b>BLID</b>	hsa-miR-143	1	0	0	0	1
<b>BLID</b>	hsa-miR-145	1	0	0	0	1
<b>CALD1</b>	hsa-miR-196a	1	0	1	1	3
<b>CALD1</b>	hsa-miR-1290	1	0	1	1	3
<b>CALD1</b>	hsa-let-7c	1	0	1	1	3
<b>CALD1</b>	hsa-miR-409	1	0	0	1	2
<b>CALD1</b>	hsa-miR-143	1	0	0	0	1
<b>CALD1</b>	hsa-miR-373	1	0	0	0	1
<b>CALD1</b>	hsa-miR-373	1	0	0	0	1
<b>CALD1</b>	hsa-miR-409	1	0	0	0	1
<b>COL1A1</b>	hsa-let-7c	1	1	1	1	4
<b>COL1A1</b>	hsa-miR-196a	1	0	1	1	3
<b>COL1A1</b>	hsa-miR-1260	1	0	1	1	3
<b>COL1A1</b>	hsa-miR-100	1	0	1	0	2
<b>COL1A1</b>	hsa-miR-373	1	0	1	0	2
<b>COL1A1</b>	hsa-miR-145	1	0	0	0	1
<b>COL1A2</b>	hsa-miR-196a	1	0	1	1	3
<b>COL1A2</b>	hsa-let-7c	1	0	1	1	3
<b>COL1A2</b>	hsa-miR-100	1	0	0	0	1
<b>COL1A2</b>	hsa-miR-143	1	0	0	0	1
<b>COL1A2</b>	hsa-miR-373	1	0	0	0	1
<b>COL1A2</b>	hsa-miR-409	1	0	0	0	1
<b>COL3A1</b>	hsa-miR-196a	1	1	1	1	4
<b>COL3A1</b>	hsa-let-7c	1	1	1	1	4
<b>COL3A1</b>	hsa-miR-100	1	0	1	0	2
<b>COL3A1</b>	hsa-miR-1290	1	0	0	0	1
<b>CTSZ</b>	hsa-miR-1260	1	0	1	0	2
<b>CTSZ</b>	hsa-miR-143	1	0	0	0	1
<b>DCN</b>	hsa-let-7c	1	0	1	1	3
<b>DCN</b>	hsa-miR-100	1	0	1	0	2
<b>DCN</b>	hsa-miR-373	1	0	1	0	2
<b>DCN</b>	hsa-miR-409	1	0	0	1	2
<b>DCN</b>	hsa-miR-373	1	0	1	0	2
<b>DCN</b>	hsa-miR-196a	1	0	0	0	1
<b>DCN</b>	hsa-miR-1290	1	0	0	0	1

Gene	miRNA	miRanda	miRDB	miRWalk	Target scan	SUM
DCN	hsa-miR-373	0	0	1	0	1
DCN	hsa-miR-373	0	0	1	0	1
DCN	hsa-miR-373	0	0	1	0	1
DCN	hsa-miR-145	0	0	0	1	1
DKK3	hsa-miR-1290	1	1	1	1	4
DKK3	hsa-let-7c	1	0	1	1	3
DKK3	hsa-miR-1290	0	0	1	1	2
DKK3	hsa-miR-196a	1	0	1	0	2
DKK3	hsa-miR-373	1	0	1	0	2
DKK3	hsa-miR-373	0	0	1	0	1
EMP1	hsa-miR-1290	1	0	1	1	3
EMP1	hsa-let-7c	1	0	1	0	2
EMP1	hsa-miR-143	1	0	1	0	2
EMP1	hsa-miR-373	1	0	1	0	2
EMP1	hsa-miR-145	1	0	0	1	2
EMP1	hsa-miR-1260	1	0	0	1	2
EMP1	hsa-miR-409	1	0	0	0	1
EPB41L2	hsa-miR-196a	1	0	0	1	2
EPB41L2	hsa-miR-409	1	0	0	1	2
EPB41L2	hsa-miR-145	1	0	0	0	1
EPB41L2	hsa-miR-373	1	0	0	0	1
ESR1	hsa-miR-196a	1	0	1	1	3
ESR1	hsa-miR-145	1	0	1	1	3
ESR1	hsa-miR-373	1	0	1	1	3
ESR1	hsa-miR-196b	1	0	1	1	3
ESR1	hsa-miR-409	1	0	1	1	3
ESR1	hsa-miR-1290	1	0	1	1	3
ESR1	hsa-miR-373	0	1	1	1	3
ESR1	hsa-let-7c	1	0	1	0	2
ESR1	hsa-miR-145	0	0	1	1	2
ESR1	hsa-miR-373	0	0	1	1	2
ESR1	hsa-miR-1290	0	0	1	1	2
ESR1	hsa-miR-143	1	0	1	0	2
ESR1	hsa-miR-373	1	0	1	0	2
ESR1	hsa-miR-373	0	0	1	1	2
ESR1	hsa-miR-1290	0	0	1	1	2
ESR1	hsa-miR-143	0	0	1	0	1
ESR1	hsa-miR-373	0	0	1	0	1
ESR1	hsa-miR-100	1	0	0	0	1
ESR1	hsa-miR-373	0	0	1	0	1
ESR1	hsa-miR-143	0	0	1	0	1

Gene	miRNA	miRanda	miRDB	miRWalk	Target scan	SUM
<b>ESR1</b>	hsa-miR-373	0	0	1	0	1
<b>FBN1</b>	hsa-miR-100	1	0	1	0	2
<b>FBN1</b>	hsa-miR-143	1	0	1	0	2
<b>FBN1</b>	hsa-miR-196a	1	0	0	1	2
<b>FBN1</b>	hsa-miR-409	1	0	0	1	2
<b>FBN1</b>	hsa-miR-1290	1	0	0	1	2
<b>FBN1</b>	hsa-let-7c	1	0	0	0	1
<b>FKBP10</b>	hsa-miR-1290	1	0	1	1	3
<b>FKBP10</b>	hsa-miR-1260	1	0	1	1	3
<b>FKBP10</b>	hsa-let-7c	1	0	1	1	3
<b>FKBP10</b>	hsa-miR-99a	1	0	1	0	2
<b>FKBP10</b>	hsa-miR-196a	1	0	0	0	1
<b>FKBP10</b>	hsa-miR-143	1	0	0	0	1
<b>FSTL1</b>	hsa-miR-409	1	0	1	1	3
<b>FSTL1</b>	hsa-miR-100	1	0	1	0	2
<b>FSTL1</b>	hsa-miR-373	1	0	1	0	2
<b>FSTL1</b>	hsa-miR-1290	1	0	0	1	2
<b>FSTL1</b>	hsa-miR-1260	1	0	0	1	2
<b>FSTL1</b>	hsa-let-7c	1	0	0	0	1
<b>FSTL1</b>	hsa-miR-373	1	0	0	0	1

Potentially regulating miRNAs were predicted by 4 different algorithms from the miRWalk 2.0 database. 0 indicates not identified, whereas 1 indicates the identified miRNA

### 4.2.3.2 The Expression of identified miRNAs in Osteosarcoma Cell

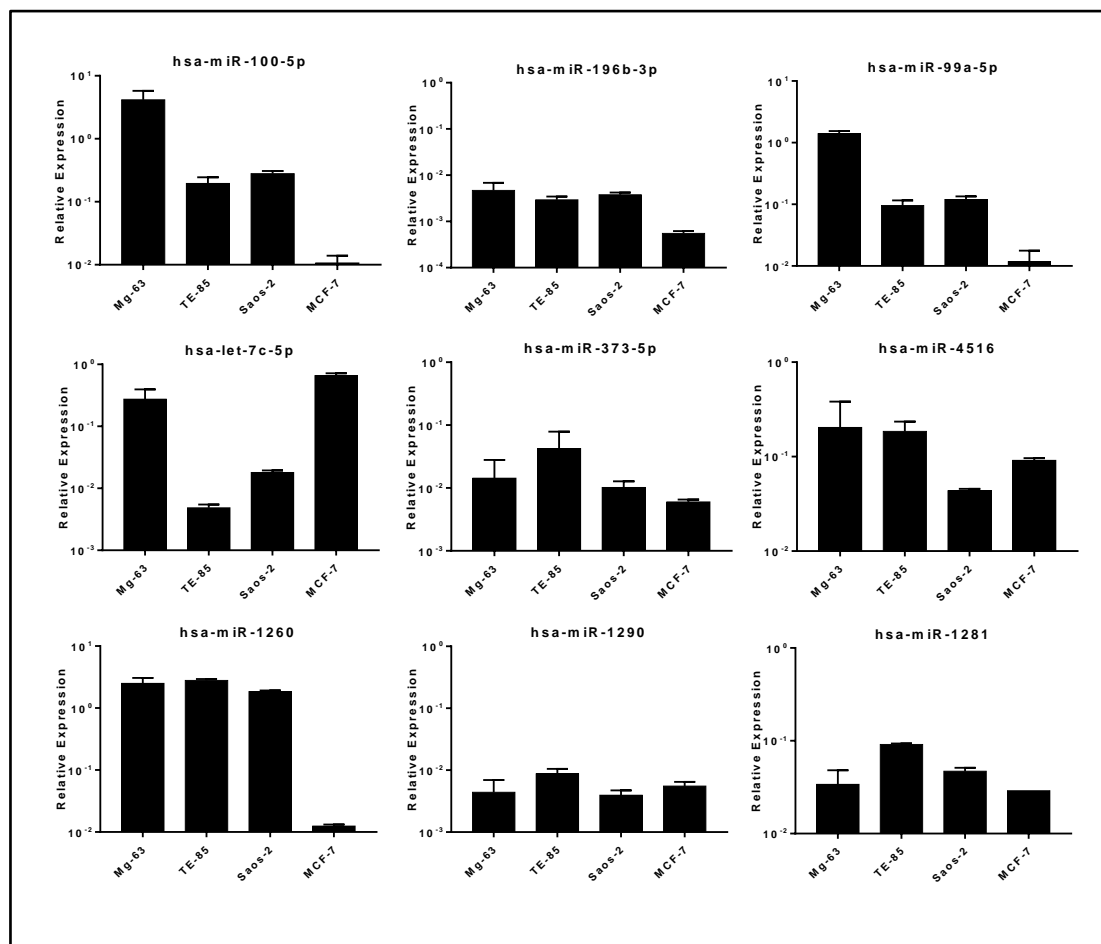
#### Lines

Twelve of the bioinformatically-predicted target miRNAs (previous section) were selected because they were identified as differentially expressed between osteopenia and osteoporosis compared to non-osteoporosis female (>40 years) serum RNA pools using QIAGEN miRNA PCR Array. These 12 miRNAs were hsa-miR-100-5p, hsa-miR-373-5p, hsa-miR-4516, hsa-miR-99a-5p, hsa-miR-196b-3p, hsa-miR-143-3p, hsa-miR-409-3p, hsa-miR-145-3p, has-miR-1260, has-miR-1281, has-miR-1290 and hsa-let-7c-5p.

3 miRNAs, has-miR-143-3p, has-miR-409-3p and has-miR-145 were not detected in the osteosarcoma cells by RT-qPCR. The levels of four miRNAs, miR-100-5p, miR-99a-5p, miR-196b-3p and let-7c-5p were lower in the TE-85 cells compared to the Mg-63 and Saos-2 cells (**Figure 4-11**). The expression levels of miR-100-5p in Mg-63 and Saos-2 were 21.2 and 1.5- fold higher compared to its level in TE-85. Also, the expression levels of miR-196b-3p in Mg-63 and Saos-2 were 1.6 and 1.3-fold higher than that in TE-85. miR-99a-5p was highly expressed in Mg-63 and Saos-2 with levels of 14.6 and 1.2-fold respectively than that in TE-85. The expression levels of miR-let-7c-5p in Mg-63 and Saos-2 were 56 and 3.6- fold higher compared to its level in TE-85.

The levels of four miRNAs, miR-373-5p, miR-4516, miR-1260 and miR-1290 were lower in Saos-2 compared to those in Mg-63 and TE-85( **Figure 4-11**). The expression levels of miR-373-5p in Mg-63 and TE-85 were 1.4 and 4.2- fold higher compared to its level in Saos-2. miR-4516 was highly expressed in Mg-63 and TE-85 with levels of 4-fold than that in Saos-2. Also, the expression levels of miR-1260 in Mg-63 and TE-85 were 1.3 and 1.4-fold higher than that in Saos-2. The expression levels of

miR-1290 in Mg-63 and TE-85 were 1.1 and 2.2- fold higher compared to its level in Saos-2. The levels of miR-1281 were lower in Mg-63 compared to those in TE-85 and Sao-2 by 1 and 2.7-fold respectively (**Figure 4-11**). Overall, the RT-qPCR data showed differences in the expression of those miRNAs in the three osteosarcoma cell lines in the normal condition.



**Figure 4-11: The expression of different miRNAs in osteosarcoma cell lines and MCF-7 cells**

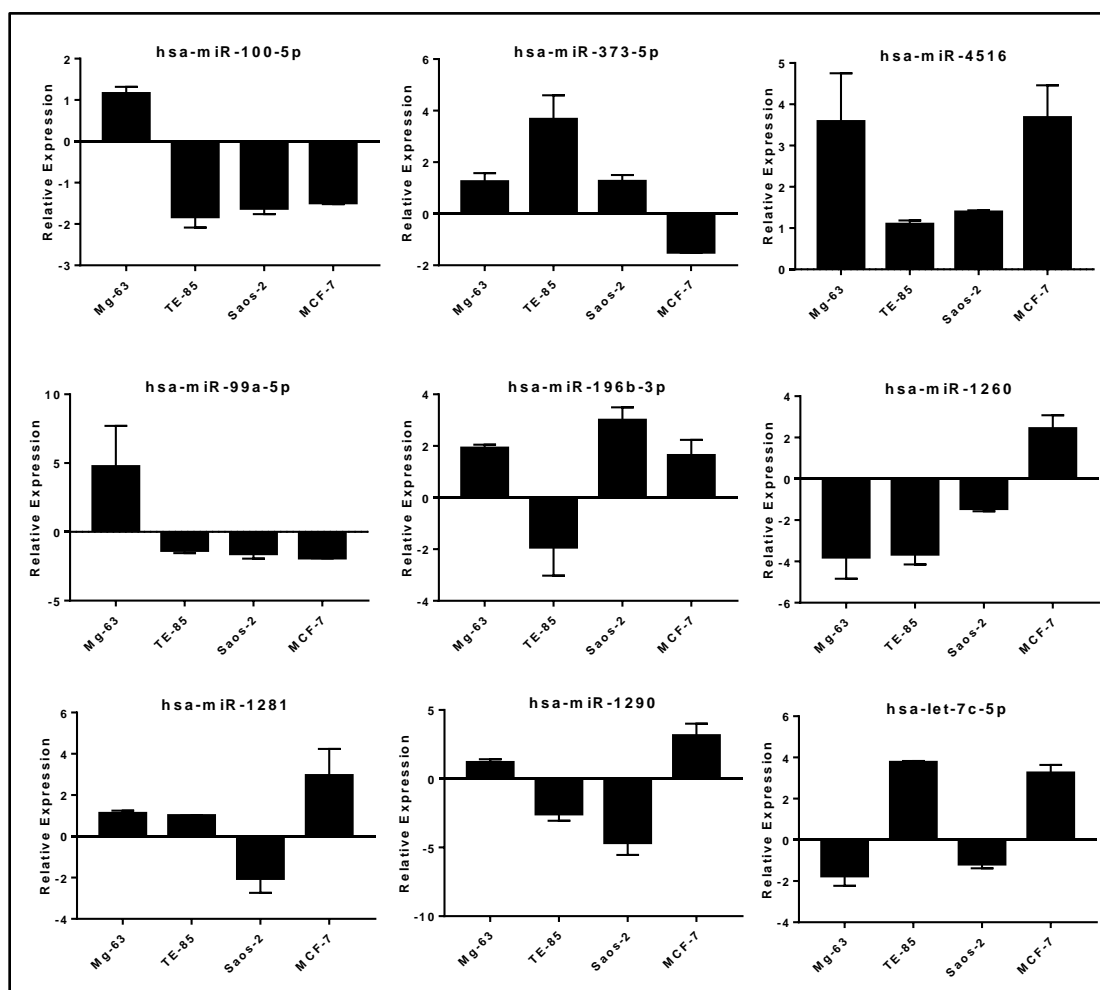
The relative expression of 12 selected miRNAs was determined by the relative Ct method normalized to SNORD96A (Data presented as Log 10 of  $2^{-\Delta Ct}$ ). Each bar represents the mean and  $\pm$ SD (n=3).

### 4.2.3.3 Estrogen-responsiveness of miRNAs in Osteosarcoma Cell Lines

In order to study the effect of  $\beta$ -estradiol on the 9 miRNAs tested previously, RT-qPCR was carried out for the three osteosarcoma cell lines and MCF-7 cells after treatment with 1 nM  $\beta$ -estradiol compared to the control incubated in the absence of  $\beta$ -estradiol. Relative levels of miRNAs were determined using the  $2^{-\Delta\Delta Ct}$  method with SNORD96A as a housekeeping control gene. The RT-qPCR data are summarized in **Figure 4-12** and **Table 4-3**.

MiR-100-5p exhibited lower expression in the three osteosarcoma cell lines, Mg-63, TE85 and Saos-2 upon estrogen treatment compared to the control non-treated those osteosarcoma cells ( $< \pm$  two-fold change).  $\beta$ -estradiol increase the expression of miR-373-5p and miR-let-7c-5p in TE-85 by 3.6 and 3.8-fold respectively compared to non-treating control, but these miRNAs were not changed in Mg-63 and Saos-2. However,  $\beta$ -estradiol increase the expression of miR-4516 and miR99a-5p in Mg-63 by 3.6 and 4.7-fold respectively compared to non-treating control, but these miRNAs were not changed in TE-85 and Saos-2. The level of miR-196b-3p were increased in Mg-63 and Saos-2 by 2 and 3-fold, but decreased in TE-85 by 2-fold upon estrogen treatment compared to the control non-treated. The RT-qPCR data revealed that  $\beta$ -estradiol decrease the expression of miR-1260 in Mg-63 and TE-85 compared to non-treating control by 3.8 and 3.7-fold respectively, but this miRNA was not changed in Saos-2 cells. RT-qPCR data showed that  $\beta$ -estradiol decrease the level of miR-1290 in TE-85 and Saos-2 compared to non-treating control by 2.6 and 4.6-fold respectively, but this miRNA was not changed in Mg-63. The expression level of miR-1281 was decreased upon  $\beta$ -estradiol treatment compared to non-treated control only in Saos-2 by 2-fold. Therefore, although these

miRNAs were differentially expressed between the three osteosarcoma cells, they were also found to be estrogen related miRNAs. Thus, in the reduced estrogen in the postmenopausal osteoporosis women, the level of these miRNAs will change which will then provide an explanation of the gene changes seen in the mRNAs because these miRNAs were originally selected as being regulators of the differentially-expressed mRNAs.



**Figure 4-12: RT-qPCR result of treated osteosarcoma cell line and MCF-7 with 1nM  $\beta$ -estradiol of some miRNAs**

Fold Change of genes were determined by the relative Ct method normalized to SNORD96A ( $2^{-\Delta\Delta Ct}$ ). Each horizontal line represents the mean and  $\pm$ SD of triplicated counting.

**Table 4-3: The effect of  $\beta$ -estradiol on the expression of miRNAs in osteosarcoma cell lines**

ID	miRNAs	Fold change in Mg-63	Fold change in TE-85	Fold change in Saos-2	Fold change in MCF-7
1	hsa-miR-100-5p	$1.16 \pm 0.17$	$-1.83 \pm 0.27$	$-1.62 \pm 0.15$	$-1.49 \pm 0.03$
2	hsa-miR-373-5p	$1.25 \pm 0.33$	$3.66 \pm 0.93$	$1.26 \pm 0.26$	$-1.49 \pm 0.03$
3	hsa-miR-4516	$3.58 \pm 1.17$	$1.10 \pm 0.09$	$1.4 \pm 0.04$	$3.68 \pm 0.79$
4	hsa-miR-99a-5p	$4.74 \pm 2.9$	$-1.36 \pm 0.21$	$-1.58 \pm 0.37$	$-1.89 \pm 0.03$
5	hsa-miR-196b-3p	$1.93 \pm 0.14$	$-1.94 \pm 1.08$	$3 \pm 0.5$	$1.63 \pm 0.61$
6	hsa-miR-1260	$-3.8 \pm 1.05$	$-3.65 \pm 0.5$	$-1.44 \pm 0.14$	$2.43 \pm 0.66$
7	hsa-miR-1281	$1.12 \pm 0.13$	$1.01 \pm 0.01$	$-2.04 \pm 0.7$	$2.95 \pm 1.3$
8	hsa-miR-1290	$1.19 \pm 0.23$	$-2.56 \pm 0.5$	$-4.64 \pm 0.91$	$3.13 \pm 0.88$
9	hsa-let-7c-5p	$-1.76 \pm 0.48$	$3.76 \pm 0.06$	$-1.18 \pm 0.22$	$3.25 \pm 0.39$



## 4.2.4 Identification of Differentially Expressed mRNAs/miRNAs in Osteoporosis Clinical Specimens

The major objective of this part is to find out if the  $\beta$ -estradiol related mRNAs/miRNAs identified in the cells lines change in postmenopausal osteoporosis compared to non-osteoporotic controls caused by reduced estrogen. The level of expression of the estrogen related mRNAs/miRNAs identified in the osteosarcoma cells above were studied by RT-qPCR in individual peripheral blood mononuclear cells (PBMC), which are osteoclast precursor cells (Fujikawa et al., 1996), obtained from participants in a blood sample. These participants were recruited from referrals to the Radiology Department for a bone density scan at the Royal Liverpool University Hospital. Bone mineral density (BMD) and T-Score were obtained. Blood samples were taken and PBMCs were isolated (Material and Methods, Section 2.9). Participants were categorised into three groups, based on their T-Score. A non-osteoporotic (NOPC) group having T score  $>-1.0$ , an osteopenia (OPA) group with T score  $<-1.0$  and  $>-2.5$  and an osteoporosis (OPS) group with T score  $\leq -2.5$ .

A total of 100 participants were recruited, 82 participants were female and 18 were male as summarized in **Table 4-4**. The cut-off age was 40 years, so the 3 participants below 40 was excluded. There was no significant age mean difference between the three study groups, (p value = 0.078). Thirteen percent of the recruits were non-osteoporotic (10 female and 3 male), sixty five percent of the participants were osteopaenia (54 female and 11 male) and twenty two percent were osteoporosis (18 female and 4 male). Total RNA was prepared from PBMC cells isolated from blood samples, and subjected to RT-qPCR as described in (Material and Methods, Section 2.10). RT-qPCR analysis

was carried out with GAPDH levels used as an internal control for real-time PCR. All q-PCR data were logarithmically transformed.

**Table 4-4: Characteristics of clinical samples (F=82, M= 18)**

Condition	Sex (No)	Age (year) mean $\pm$ SD	No. of Fracture (%)	BMD g/cm <sup>2</sup> Mean $\pm$ SD	T-Score Lumbar Spine (L2-L4) Mean $\pm$ SD
<b>Non-osteoporotic</b>	F (n=10)	68 $\pm$ 9.8	3 (30%)	1 $\pm$ 0.1	0.8 $\pm$ 1.5
	M (n=3)	45.3 $\pm$ 25.7	1 (30%)	0.3 $\pm$ 0.6	0.3 $\pm$ 0.6
	Total (n=13)	59.2 $\pm$ 19.9	4 (30%)	0.8 $\pm$ 0.4	0.7 $\pm$ 1.3
<b>Osteopenia</b>	F (n=54)	63.9 $\pm$ 10.3	25 (50%)	0.8 $\pm$ 0.2	-1.2 $\pm$ 0.9
	M (n=11)	65.5 $\pm$ 13.4	3 (30%)	0.8 $\pm$ 0.3	-0.7 $\pm$ 1.4
	Total (n=65)	64.2 $\pm$ 10.7	28 (40%)	0.8 $\pm$ 0.2	-1.1 $\pm$ 1
<b>Osteoporosis</b>	F (n=18)	68.8 $\pm$ 14.1	8 (40%)	0.7 $\pm$ 0.2	-2.6 $\pm$ 0.9
	M (n=4)	67.3 $\pm$ 10.2	1 (30%)	0.6 $\pm$ 0.1	-2.6 $\pm$ 1
	Total (n=22)	68.05 $\pm$ 12.1	9 (35%)	0.7 $\pm$ 0.2	-2.6 $\pm$ 0.95

#### 4.2.4.1 Differentially Expressed mRNAs Associated with Osteoporosis

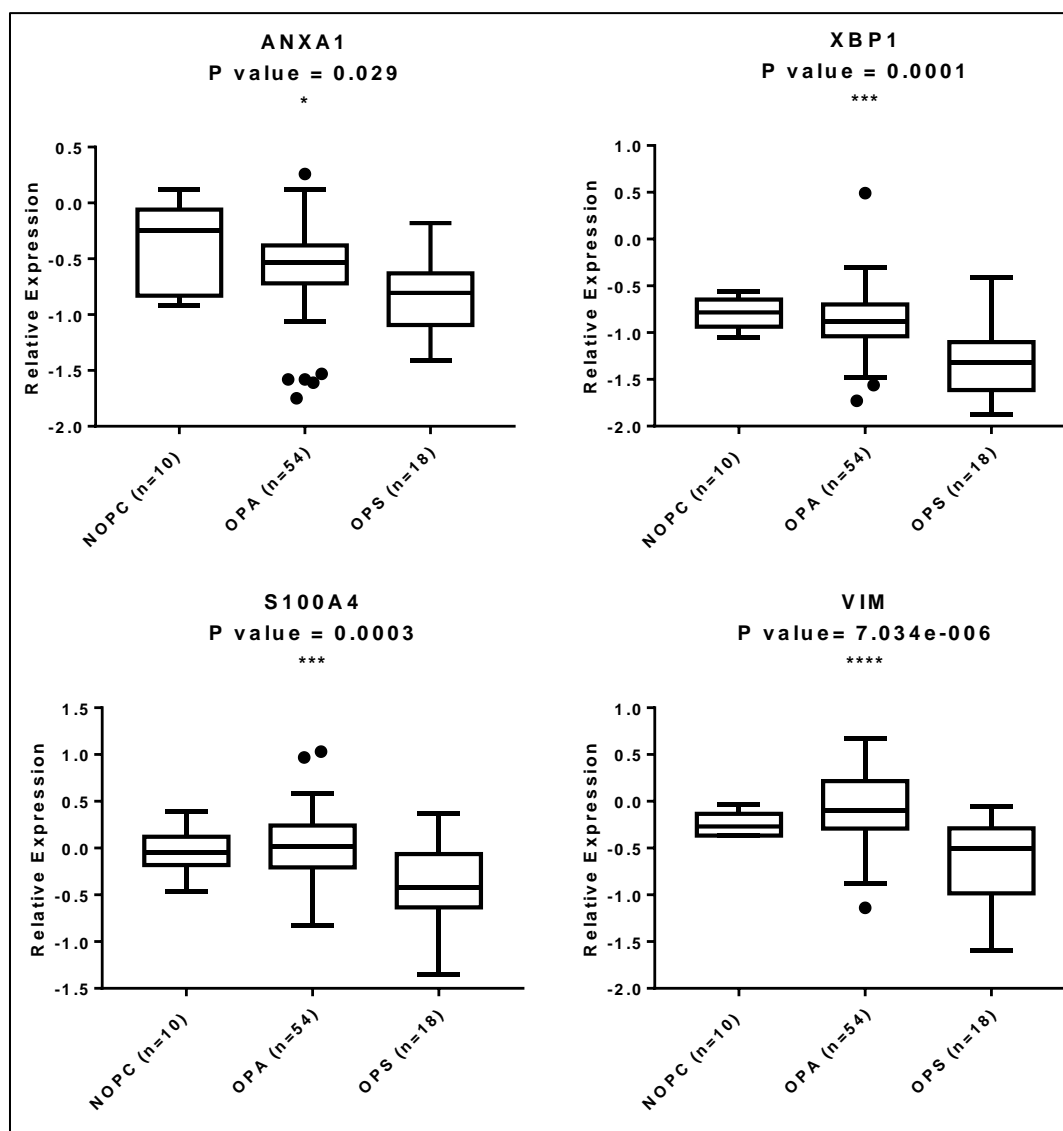
##### Patients

To find out whether any of the 45 mRNAs identified (Section 4.1.3) as  $\beta$ -estradiol related genes in osteosarcoma cell were differentially expressed in PBMC cells of patients in the three patient groups above, a pilot experiment on 4 female samples from each group was carried out by RT-qPCR. Out of the 45 mRNAs, 9 mRNAs (ALDH3A1, BLID, CALD1, COL1A1, COL1A2, COL3A1, IFI27, TFAP2C and S100A2) were not detected in the PBMC samples and 22 genes showed a Ct of  $>30$ . Thus, these 31 genes were excluded from further study. The remaining 14 genes showed Ct value of less than 30 on the 4 PBMC samples. These genes were ANXA1, CTSZ, VIM, IFI16, TMEM64, TRIM37, TGF $\beta$ I, TRAM2, GSDMD, KRT8, NFKBIA, S100A4, XBP1 and RUNX2.

The mean expression of all 14 mRNAs were compared in each patient between the three groups. One-way ANOVA (Bonferroni's multiple comparisons test) was used to

compare the mean of the expression of each gene between the three groups, NOPC, OPA and OPS. The RT-qPCR  $2^{-\Delta Ct}$  data were logarithmically transformed (Log10) since it provides a symmetrical data to show more accurate and relevant interpretation (Schmittgen and Livak, 2008).

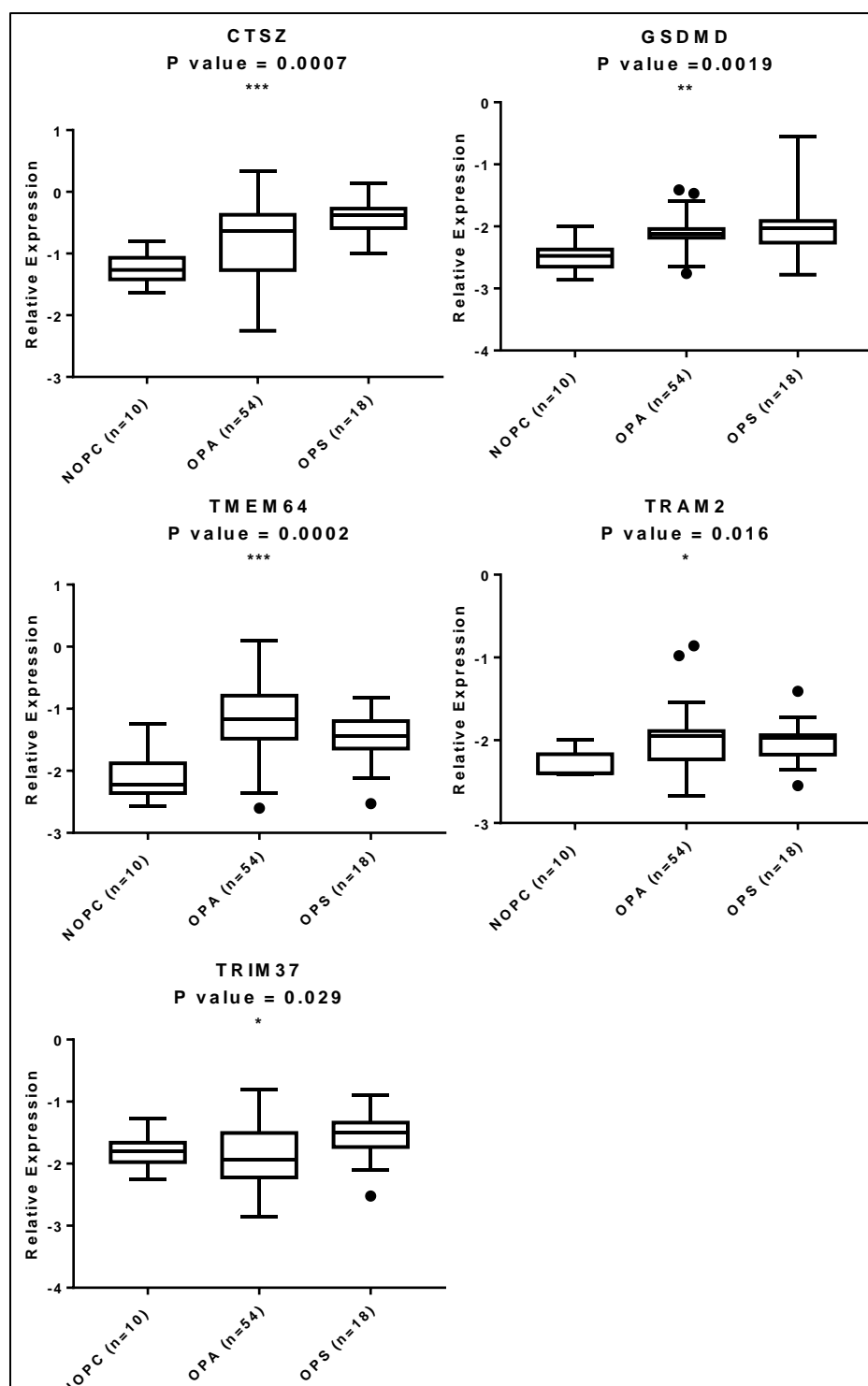
The expression levels of ANXA1 mRNA were much lower in osteoporotic patients compared with non-osteoporotic control and osteopenia patients (ANOVA  $p = 0.029$ , **Figure 4-13**). There was a significant difference between non-osteoporotic group and osteoporosis group (Bonferroni's  $p = 0.021$ ). The levels of XBP1 mRNA were much lower in osteoporotic patients compared with osteopenia patients and non-osteoporotic controls (ANOVA  $p = 0.0001$ , Figure 4-13), and there was a significant decrease in the expression of XBP1 mRNA in the osteoporosis compared to the non-osteoporotic control (Bonferroni's  $p = 0.0013$ ). The differences of S100A4 mRNA expression between the three groups showed a significant change (ANOVA  $p = 0.0003$ , Figure 4-13). The q-PCR data showed that there was no change in osteopenia samples but a significant decrease in the osteoporosis sample once compared to non-osteoporotic samples (Bonferroni's  $p = 0.023$ ). The levels of VIM mRNA were significantly lower in the osteoporosis group compared to non-osteoporotic participants and osteopenia patients (ANOVA  $p = <0.0001$ , Figure 4-13) and there was a significant decrease in the expression of VIM in the osteoporosis patient non-osteoporotic (Bonferroni's  $p = 0.042$ ). The results revealed that the levels of two mRNAs, ANXA1, and XBP1 in PBMCs were much lower in osteoporosis patients compared with osteopenia patients and non-osteoporotic group (ANOVA  $p = 0.029$ ,  $p = 0.0001$ , respectively).



**Figure 4-13: Decreased mRNAs expression in PBMCs of osteoporosis patients**

RT-qPCR data showed that the expression of ANXA1, XBP1, S100A4 and VIM were significantly lower in OPS compared to NOPC. box-and-whisker plots showed the expression of mRNA in non-osteoporotic, osteopenia and osteoporosis groups. p value was calculated using one-way ANOVA. Box plots showing 25th, 50th, and 75th percentiles (horizontal bars) and minimum to maximum ranges (error bars). Dots indicate outliers

The levels of CTSZ mRNA were much higher in osteoporotic patients compared with osteopenia patients and non-osteoporotic controls (ANOVA  $p = 0.0007$ , **Figure 4-14**). The significant difference increases in CTSZ mRNA level occurred between CTSZ expression in the non-osteoporotic and the osteopaenia groups and between the non-osteoporotic and osteoporosis groups was increased significantly (Bonferroni's  $p = 0.046$  and  $0.0004$ , respectively). The levels of GSDMD mRNA were higher in osteoporosis patients compared with osteopenia patients and the non-osteoporotic control (ANOVA  $p = 0.0019$ , **Figure 4-14**). There was a significant increase in GSDMD mRNA expression levels in the osteopaenia and osteoporosis samples compared to that in the non-osteoporotic samples (Bonferroni's  $p = 0.004$ ,  $0.0011$  respectively). The levels of TMEM64 mRNA were higher in both osteopenia and osteoporosis patients compared to non-osteoporotic control (ANOVA  $p = 0.0002$ ). There was significantly increased (Bonferroni's  $p = 0.0002$  and  $p = 0.027$ , **Figure 4-14**) in the expression of TMEM64 mRNA in the osteopaenia and osteoporosis samples compared to that in the non-osteoporotic samples. The levels of TRAM2 mRNA was significantly higher in the osteopenia and osteoporosis patients compared to that in the non-osteoporotic control (ANOVA  $p = 0.016$ , **Figure 4-14**). The levels of TRIM37 mRNA was significantly higher in the osteoporosis patients compared to that in the non-osteoporotic control (ANOVA  $p = 0.029$ , **Figure 4-14**). The results also showed that the levels of three mRNAs, CTSZ, GSDMD and TMEM64 in PBMC were significantly increased in both osteopaenia and osteoporosis compared to the non-osteoporotic group (ANOVA  $p = 0.0007$ ,  $p = 0.0019$ , and  $p = 0.0002$  respectively).



**Figure 4-14: An increase of mRNAs expression in PBMCs of osteoporosis patients**  
RT-qPCR data showed that the expression of CTSZ, GSDMD, and TMEM64 were significantly higher in OPS compared to NOPC. box-and-whisker plots showing the expression of mRNA in non-osteoporotic, osteopenia and osteoporosis groups. p value was calculated using one-way ANOVA. Box plots show 25th, 50th, and 75th percentiles (horizontal bars) and minimum to maximum ranges (error bars). Dots indicate outliers

#### 4.2.4.2 Differentially Expressed miRNAs Associated with Osteoporosis Patients

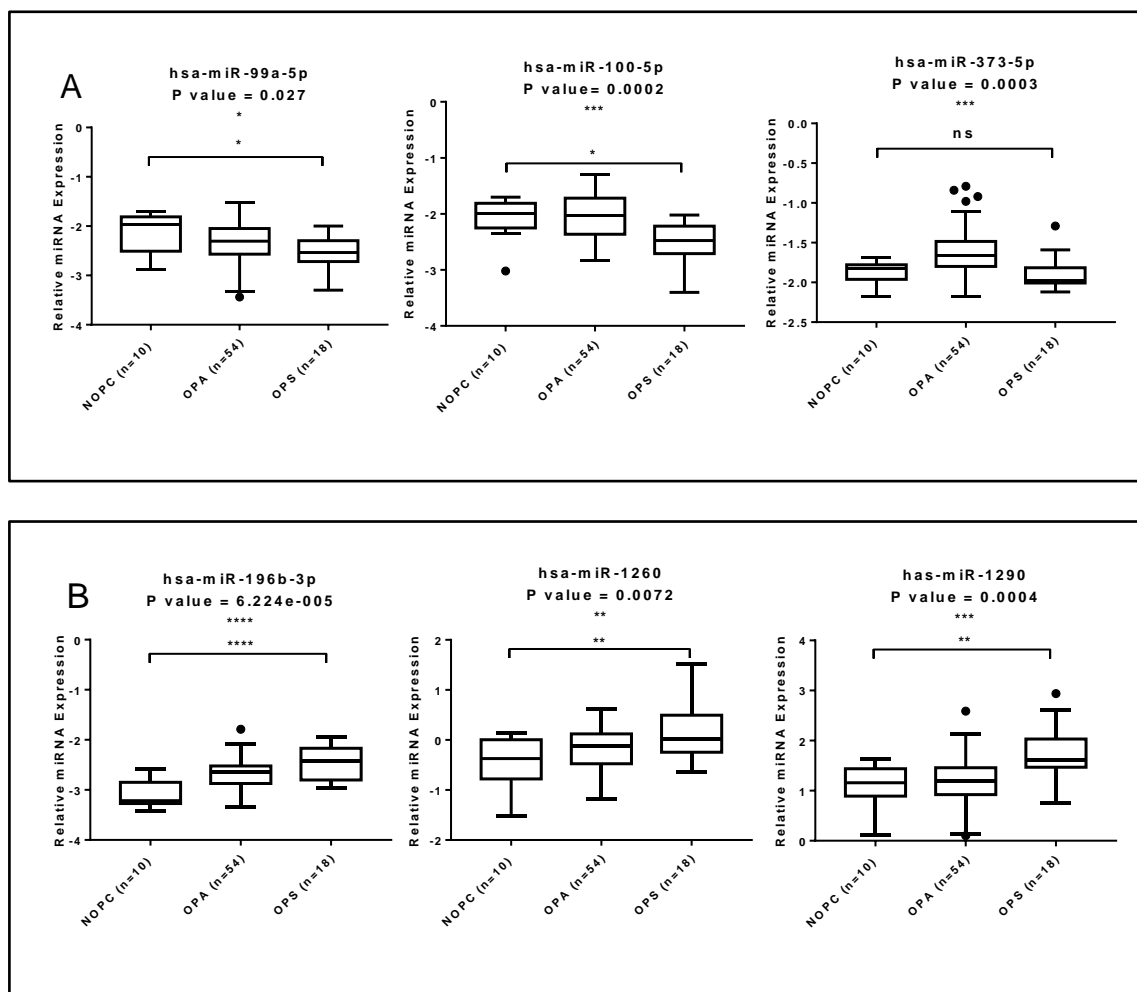
To find out whether any of the 9 miRNAs (Section 4.1.3) identified as  $\beta$ -estradiol related miRNAs in osteosarcoma cells were differentially expressed in PBMC cells of patients in the three patient groups, a pilot experiment on 4 female samples from each group was carried out by RT-qPCR. Two out of nine differentially expressed miRNAs identified from pilot experiments were excluded, has-miR-1281 had Ct >31, and has-miR-let-7c-5p did not showed any changed expression between the three groups, (ANOVA  $p = 0.42$ ). The remaining 7 miRNAs showed Ct value that were less than 30 on the 4 PBMC samples were tested on all participants' samples. These miRNAs are hsa-miR-100-5p, hsa-miR-373-5p, hsa-miR-4516, hsa-miR-99a-5p, hsa-miR-196b-3p, has-miR-1260, and has-miR-1290.

The RT-qPCR data revealed that the levels of miRNA-99a-5p were lower in osteoporosis patients compared with osteopenia patients and non-osteoporotic control (ANOVA  $p = 0.027$ , **Figure 4-15A**). A significantly difference was shown between the non-osteoporotic group and the osteoporosis group (Bonferroni's  $p = 0.026$ ). Similarly, the expression of miR-100-5p was lower in osteoporotic patients compared with osteopenia patients and non-osteoporotic controls (ANOVA  $p = 0.0002$ , **Figure 4-15A**). There was also significantly difference shown between non-osteoporotic group and osteoporosis group, (Bonferroni's  $p = 0.015$ ). RT-qPCR data showed that the level of hsa-miR-373-5p in non-osteoporotic participants was significantly different between that in osteopaenia and osteoporosis patients (ANOVA  $p = 0.0003$ ), but no change was seen between non-osteoporotic control and osteoporosis patients ( $p > 0.9$ , **Figure 4-15A**).

Overall, the results revealed that the levels of two miRNAs, miRNA-99a-5p, and miR-100-5p in PBMCs were much lower in osteoporosis patients compared with osteopenia patients and non-osteoporotic group (ANOVA  $p = 0.027$ ,  $p = 0.0002$  respectively).

On the other hand, the RT-qPCR data showed that the expression levels of miR-196b-3p was much higher in osteoporotic patients compared with osteopenia patients and non-osteoporotic controls (ANOVA  $p = <0.0001$ , **Figure 4-15B**). There was a significant increase in miR-196b-3p mRNA expression in the osteopaenia and osteoporosis samples compared to that in the non-osteoporotic samples (Bonferroni's  $p = 0.0008$ ,  $p < 0.0001$ ). Moreover, the expression level of hsa-miR-1260 and hsa-miR-1290 were higher in osteoporosis patients compared with osteopenia patients and the non-osteoporotic control (ANOVA  $p = 0.0072$  and  $p = 0.0004$ , respectively, **Figure 4-15B**). The expression level of hsa-miR-1260 and hsa-miR-1290 in osteoporosis participants was significantly higher compared to those in non-osteoporotic participants (Bonferroni's  $p = 0.0062$  and  $0.0058$  respectively, **Figure 4-15B**). Thus, the results revealed that the levels of three miRNAs, miRNA-196b-3p, miR-1260 and miR-1290 in PBMCs were much higher in osteoporosis patients compared with osteopenia patients and non-osteoporotic group (ANOVA  $p = <0.0001$ ,  $p = 0.0072$  and  $p = 0.0004$  respectively).





**Figure 4-15: Relative miRNA expression in PBMCs**

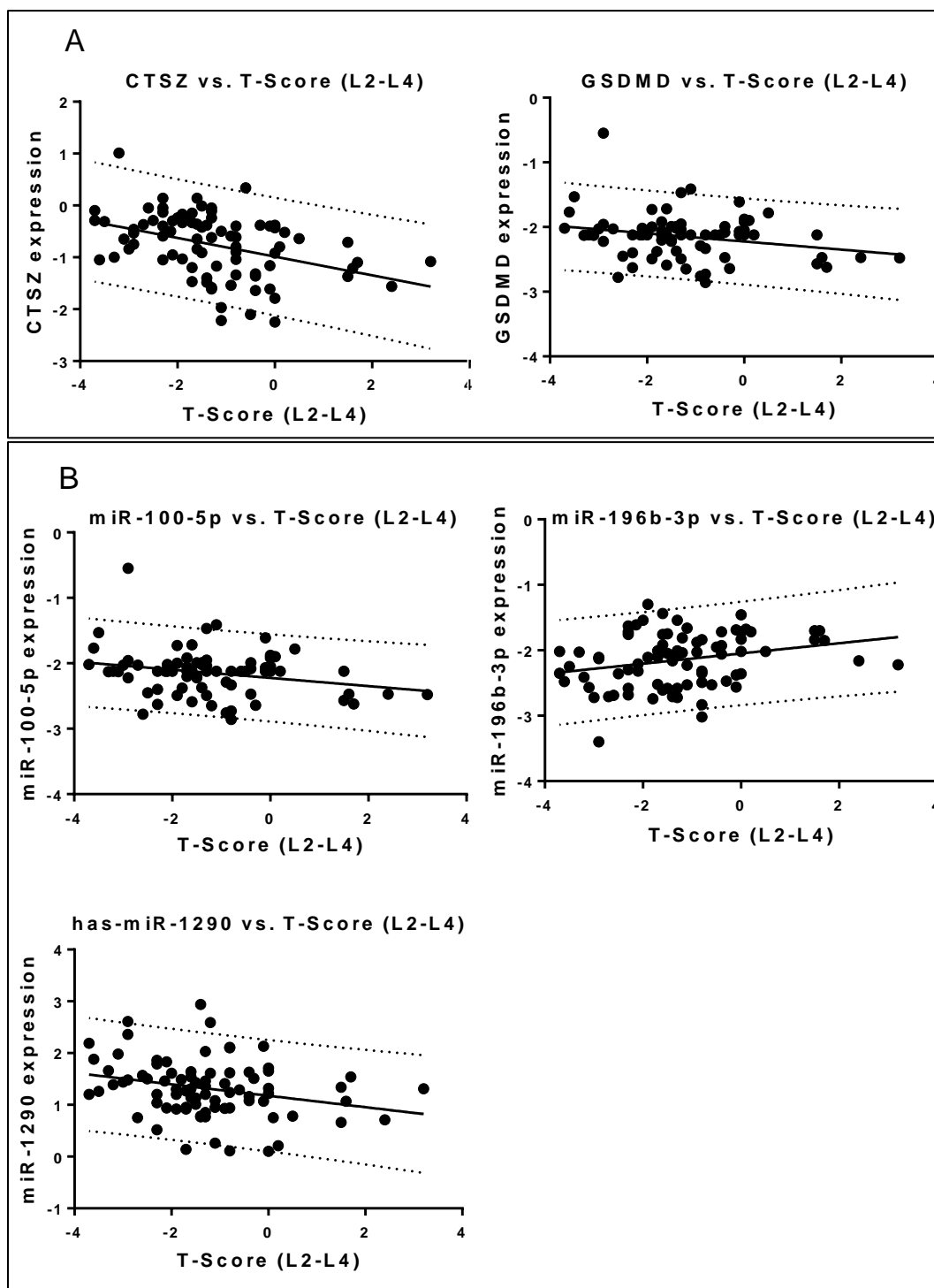
RT-qPCR data showed that (A) the expression of miRNA-99a-5p, miRNA-100-5p, and miRNA-373-5p were significantly lower in OPS compared to NOPC. (B) the expression of miRNA-196b-3p, miRNA-1260 and miRNA-1290 were significantly higher in OPS compared to NOPC. box-and-whisker plots showed the expression of miRNA in non-osteoporotic, osteopenia and osteoporosis groups. p values were calculated using one-way ANOVA. Box plots showing 25th, 50th, and 75th percentiles (horizontal bars) and minimum to maximum ranges (error bars).

#### 4.2.4.3 mRNA/miRNAs Associated with Low BMD

Pearson correlation coefficient between relative expression level and lumbar spine (L2-L4) *T*-score was calculated and tested. This was performed to verify if the levels of any particular mRNAs/miRNA correlated with the *T*-score in all the PBMC samples. Linear regression was run on each mRNA expression and *T*-score level to obtain the *T*-score correlated gene lists.

The correlation analysis revealed that the levels of CTSZ and GSDMD mRNAs in the PBMC samples significantly correlated inversely with participants' *T*-scores (Figure 4-16A,  $p = 0.0002$  and  $0.022$ , respectively). No other mRNA expression showed any significant association with the level of the *T*-score (**Table 4-5**). Therefore, the decreased level of only CTSZ and GSDMD indicates that they are associated with the reduction in bone density with osteoporosis.

The correlation analysis also demonstrated that the level of hsa-miR-100-5p, hsa-miR-196b-3p and has-miR-1290 expression in PBMC samples was significantly associated with the level of the *T*-score as illustrated in Figure 4-16B, ( $p$  value =  $0.035$ ,  $0.0004$ , and  $0.047$ ). However, the other miRNAs did not show any significant correlation between the expression level of miRNA in the PBMC of participants and their *T*-score. Although dysregulation of miR-100-5p and miR-196b-3p showed a significant correlation with the participants' *T*-score, they are not correlated with the progression of the osteoporosis. Therefore, the decreased level of only miR-1290 is suggested to be associated with the development of osteoporosis.



**Figure 4-16: T-score-associated mRNAs/miRNAs expression in PBMC samples**  
 Regression line showing the correlation between (A) mRNA and (B) miRNAs expression level and T-Score Lumbar Spine (L2-L4) in female participants. The black line denote the regression lines for samples and the 2 dots lines represent the 95% CI. Only those mRNA/miRNAs showing significant correlations are shown

**Table 4-5: Correlation of mRNAs/miRNAs expression and participants' T-score**

<b>mRNAs/miRNAs</b>	<b>Pearson r</b>	<b>P value</b>
<b>ANXA1</b>	-0.084	0.45
<b>CTSZ</b>	-0.4	0.0002
<b>VIM</b>	-0.013	0.91
<b>TMEM64</b>	-0.019	0.86
<b>TRIM37</b>	-0.152	0.17
<b>TGFB1</b>	-0.133	0.23
<b>TRAM2</b>	-0.202	0.065
<b>GSDMD</b>	-0.249	0.022
<b>NFKB1A</b>	-0.078	0.48
<b>S100A4</b>	0.032	0.77
<b>XBP1</b>	0.038	0.73
<b>miR-373-5p</b>	0.168	0.127
<b>miR-100-5p</b>	0.264	0.016
<b>miR-4516</b>	-0.068	0.538
<b>miR-99a-5p</b>	0.160	0.146
<b>miR-196b-3p</b>	-0.376	0.0004
<b>miR-1260</b>	-0.204	0.062
<b>miR-1290</b>	-0.272	0.013

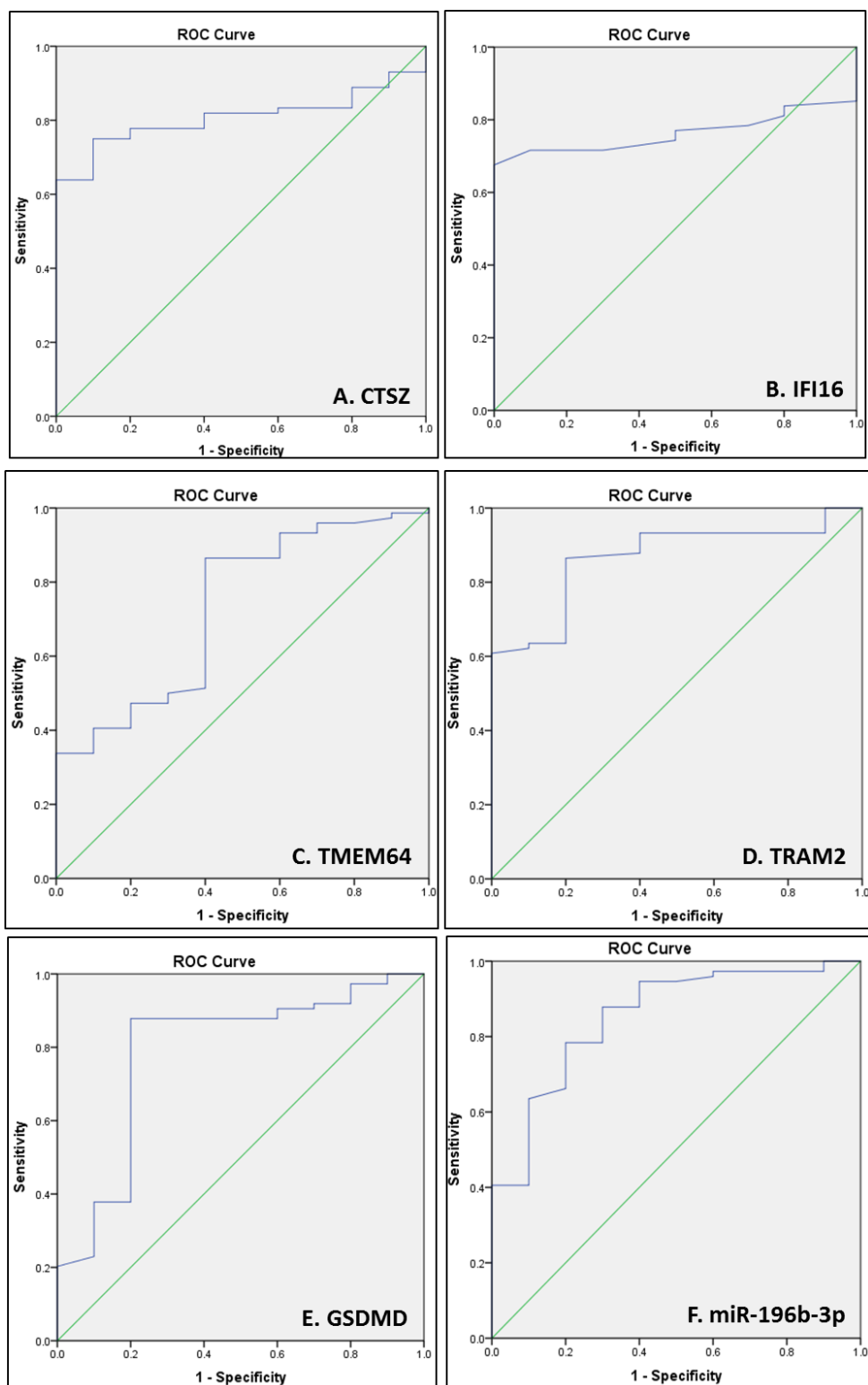
Pearson-r value in the title represents the Pearson correlation coefficient between gene expression and BMD level across all samples

#### 4.2.4.4 Diagnostic Value of mRNAs/miRNAs in Osteoporosis

AUC is the area under the receiver operating characteristic (ROC) curve. It is a graphical plot that provides the reflection of how good a test is at distinguishing between patients with disease and those without disease (Zweig and Campbell, 1993, Akobeng, 2007). There are 4 possible categories that describe the AUC values. A test with an area greater than 0.9 has high accuracy, while 0.7–0.9 indicates moderate accuracy, 0.5–0.7, shows low accuracy and <0.5 indicates a poor accuracy (Akobeng, 2007). To assess the potential diagnostic value of the significantly regulated mRNAs/miRNAs, a ROC curve analysis was performed for the relative expression of the all mRNAs/miRNAs tested in the participants' PBMC. The associated AUC was used to confirm the diagnostic value of each mRNA/miRNA. The results are summarized in **Figure 4-17A-E** and **Table 4-6**. The sensitivity and specificity associated with the optimal cut-off points are shown also in **Table 4-6**.

The AUC discrimination was significantly separated for mRNAs CTSZ, IFI16, TMEM64, TRAM2, and GSDMD. All of these five-discrimination displayed the moderate accuracy. The diagnostic value for TRAM2 and CTSZ in clinical samples for osteoporosis patient was 0.866 and 0.806 respectively, with p value of 0.0002 and 0.002, respectively. The sensitivity and specificity of TRAM2 were 86, 80 % respectively. However, the specificity was highest in CTSZ at 90% but the sensitivity was lower at 74%. Although the AUC of TMEM64 and GSDMD were significantly discriminatory, their specificity was very low, 60 and 50 % respectively. Therefore, the result suggests that only CTSZ has diagnostic value for osteoporosis.

The ROC curve analysis indicates that the level of hsa-miR-196b-3p might be a potential biomarker for distinguishing osteoporosis participants from non-osteoporotic participants',  $AUC = 0.853$ ,  $p = 0.0003$ . The sensitivity and specificity of discriminating hsa-miR-196b-3p in PBMC samples were 78% and 80% respectively. However, the rest of the mRNA/miRNA levels were not significantly separated and had a low accuracy rate. Thus, the result suggests that only hsa-miR-196b-3p might have a diagnostic value for osteoporosis.



**Figure 4-17: ROC curves of mRNAs/miRNA for osteoporosis.**

ROC curves generated from the logarithmically transformed relative expression of these mRNAs/miRNA from the participants' PBMCs tested. the result showed AUC corresponding to at least moderate accuracy.

**Table 4-6: Diagnostic value of mRNAs/miRNAs for Osteoporosis**

mRNAs/miRNAs	Area Under Curve	95% Confidence Interval		P value	Sensitivity	Specificity	Accuracy
		Lower Bound	Upper Bound				
ANXA1	0.659	0.463	0.855	0.105	85%	60%	low
<b>CTSZ</b>	<b>0.806</b>	<b>0.714</b>	<b>0.898</b>	<b>0.002</b>	<b>74%</b>	<b>90%</b>	<b>moderate</b>
VIM	0.647	0.517	0.778	0.132	69%	60%	low
<b>IFI16</b>	<b>0.763</b>	<b>0.668</b>	<b>0.858</b>	<b>0.007</b>	<b>72%</b>	<b>90%</b>	<b>moderate</b>
<b>TMEM64</b>	<b>0.730</b>	<b>0.563</b>	<b>0.896</b>	<b>0.019</b>	<b>86%</b>	<b>60%</b>	<b>moderate</b>
TRIM37	0.518	0.375	0.660	0.857	50%	50%	low
TGFBI	0.614	0.447	0.780	0.246	69%	70%	low
<b>TRAM2</b>	<b>0.866</b>	<b>0.770</b>	<b>0.961</b>	<b>0.0002</b>	<b>86%</b>	<b>80%</b>	<b>moderate</b>
<b>GSDMD</b>	<b>0.791</b>	<b>0.625</b>	<b>0.956</b>	<b>0.003</b>	<b>88%</b>	<b>50%</b>	<b>moderate</b>
KRT8	0.616	0.425	0.806	0.238	59%	80%	low
NFKBIA	0.517	0.381	0.653	0.863	49%	70%	low
S100A4	0.497	0.342	0.652	0.972	51%	70%	poor
XBP1	0.401	0.263	0.538	0.310	45%	50%	poor
<b>RUNX2</b>	0.649	0.531	0.766	0.129	57%	70%	low
miR-373-5p	0.685	0.558	0.812	0.058	57%	90%	low
miR-100-5p	0.444	0.273	0.615	0.567	45%	50%	poor
miR-4516	0.640	0.477	0.802	0.153	73%	50%	low
miR-99a-5p	0.604	0.378	0.830	0.288	66%	60%	low
miR-196b-3p	<b>0.853</b>	<b>0.731</b>	<b>0.975</b>	<b>0.0003</b>	<b>78%</b>	<b>80%</b>	<b>moderate</b>
miR-1260	0.586	0.386	0.787	0.377	58%	50%	low
miR-1290	0.555	0.374	0.736	0.576	64%	50%	low

Data were evaluated via a cut-off point which jointly maximises both sensitivity and specificity. Bold data showed the significant AUC discriminated.



### 4.3. Discussion

In this chapter, a panel of differentially expressed mRNA/miRNAs identified by RNA sequencing in Chapter 3 were analysed using cultured osteosarcoma cells and clinical samples by RT-qPCR. This was in an attempt to identify candidate mRNAs/miRNAs associated with osteoporosis patients.

Transmembrane Associated Membrane Protein 2 (TRAM2) is an estradiol-responsive protein in human fetal osteoblast cells based on case-cohort genome-wide association study (GWAS). TRAM2 suppression changed the expression of genes in pathways associated with osteoporosis risk and/or bone biology such as RANK-RANKL-OPG (Liu et al., 2014a). It has been shown to be a key activator of  $\alpha 1(I)$  collagen promoter; a downstream target of the master osteoblast differentiation factor RUNX2 (Pregizer et al., 2007). In concordance with reported findings, our data confirmed its response to  $\beta$ -estradiol in the three osteosarcoma cells and showed a significant increase in TRAM2 mRNA expression in postmenopausal osteoporosis through osteopenia as opposed to the non-osteoporotic group ( $P = 0.016$ ). Also, TRAM2 exhibited the strongest AUC discrimination at 0.866, with 86% and 80% sensitivity and specificity, respectively.

Transmembrane protein (TMEM64) is a positive regulator of osteoclast formation in mice bone marrow macrophages (BMMs). Knockdown of TMEM64 blunted the activity of osteoclast differentiation markers NFATc1, OSCAR, CTSK and PPARGC1B, and RANKL. Consistently, cells overexpressing TMEM64 showed a significant reduction in osteogenic markers including ALP, OCN, COLLA, OSX, and RUNX2 (Kim et al., 2013, Jeong et al., 2015). The expression pattern of TMEM64

mRNA PBMCs obtained from osteoporosis patients, as opposed to control PBMCs, revealed a dramatic increase with a p-value of 0.0006. Moreover, the accuracy of the diagnostic potential TMEM64 mRNA levels stands at an AUC value of 0.730 and a p-value of 0.019, which further corroborates previous findings that identify TMEM64 as a negative osteogenic protein.

Annexin A1 (ANXA1) mRNA displayed as an  $\beta$ -estradiol responsive gene by increasing the expression in Mg-63 by 3.30-fold and decreasing in TE-85 by -5.04-fold once treating with 1 nM and compare the expression against that non-treating control. The expression of ANXA1 mRNA in PBMCs was significantly decreased compared to non-osteoporotic female participants' (p = 0.029, Figure 4-14A). Several studies demonstrated the anti-inflammatory effects of Annexin 1 (ANXA 1), a calcium-dependent phospholipid-linked protein (Serhan et al., 2008, Vago et al., 2012, Lim and Pervaiz, 2007). Particularly, ANXA1 silencing reduces the rate of osteoclast formation in both arthritis and monocyte-derived human macrophages by promoting IL-6 release (Kao et al., 2014). Furthermore, ANXA1 has been shown to play a key role in bone formation in rat osteoblasts in primary cell cultures. ANXA1 enhances PTH-mediated cAMP stimulation by binding calcium ions that act as a second messenger relaying the PTH signal intracellularly (Suarez et al., 1993). In accordance with published work, our results detected reduced expression of ANXA1 in postmenopausal osteoporosis compared to non-osteoporotic participants'. This is in full agreement with the reported role of ANXA1 favoring osteoclast formation.

ANXA1 has been identified as a target for regulatory miRNAs 196a and 196b, with inversely proportional expression levels detected in a variety of cancer cell lines, in

4 cells line of each esophageal, breast, and endometrial origin (Luthra et al., 2008).

Similar correlation was also found in 10 esophageal adenocarcinoma cell line and the data of paired normal/tumor tissues from additional 10 patients (Luthra et al., 2008).

Investigations here revealed that the expression levels of ANXA1 and has-miRNA-196b-3p are inversely proportional in PBMCs, osteosarcoma cells, and MCF-7 cell line.

This further confirms that ANXA1 is inversely correlated with has-miRNA-196b-3p.

Also, two studies found that miRNA 196a inversely regulate the proliferation in human adipose-derived stem cells (hASCs); which are bone marrow-derived mesenchymal stem cells and subsequently differentiate into osteoblast (Gir et al., 2012, Kim et al., 2009).

This may correlate with this study where miRNA-196b-3p level were higher in osteoporosis PBMC cells which result in increased osteoclast activity. Also, as miRNA-100-5p is a predicted to target ANXA1, for the first time, the data confirmed that the level of miRNA-100-5p is correlated with that in ANXA1 in the PBMCs samples which is lower in osteoporosis compared to that in non-osteoporotic and osteopenia samples.

miR-100 was significantly response to  $\beta$ -estradiol with a higher level in Mg-63 cells compared to TE-85, Saos-2, and MCF-7 compared to the control of each cell. Also, female osteoporosis patients had significantly lower PBMC miR-100-5p expression levels in comparison with control samples ( $p = 0.0002$ ), which also correlated with the patients' T-score. The study by Zeng (2012) and colleagues revealed a revers association between miR-100 and osteogenic differentiation through its target gene BMPR2 in human adipose-derived mesenchymal stem cells (hASCs) *in vitro* (Zeng et al., 2012). Also, there was a significant upregulation of miR-100 expression in serum and bone tissue of osteoporotic patients compared with non-osteoporotic fracture (Seeliger et al., 2014). These results suggest that an increase in miRNA-100 expression in mature or

precursor osteoblasts, or a decrease in osteoclast precursors may be a hallmark of osteoporosis. This is mediated through either blunting osteoblast differentiation or promoting osteoclast differentiation. Therefore, taken together, the increase of miRNA-100 expression in mature or precursor osteoblasts, or a decrease in osteoclast precursors may cause bone loss and osteoporosis either by increase the osteoclast differentiation or decrease the osteoblast differentiation.

Consistent with miR-100 expression patterns, The RT-qPCR data showed miR-99a was significantly upregulated in Mg-63 cells. Also, it showed that significant downregulation of miR-99a-5p expression in PBMC of the osteoporotic samples compared to the non-osteoporotic samples, although the accuracy of the distribution was low, AUC = 0.604. Franceschetti *et al.* (2014) reported that microRNA-99b and microRNA-365 are highly increased during osteoclastogenesis. Transfection-mediated inhibition of miR-99b led to diminished size and number of osteoclasts, indicating an indispensable role of miR-99b in osteoclast formation (Franceschetti *et al.*, 2014). Altogether, our results strongly suggest that, disregarding subspecies variation, miR-99 may be involved in osteoclast formation.

Cathepsins are a family of serine, cysteine, and aspartyl protease enzymes involved in cell turnover (Santamaria *et al.*, 1998). Members of the family have been implicated in number of pathologies including cancer, neurodegenerative disorders, and arthritis (Santamaria *et al.*, 1998, Mohamed and Sloane, 2006). Of particular relevance to our studies, the association of cathepsins with bone remodelling in osteoclasts has been established (Holzer *et al.*, 2005, Lang *et al.*, 2004, Han *et al.*, 2009, Wilson *et al.*, 2009).

Furthermore, the role of CTSZ in bone disease has not been sufficiently investigated until recently. Staudt et al. (2010) reported that CTSZ secretion is detected in primary osteoblasts and osteoblastic cell lines CAL72 and Mg-63 (Staudt et al., 2010). In agreement with previous studies, we show here that the expression level of CTSZ is inversely proportional to the maturity of cultured osteosarcoma cells, (**Figure 4-3**). The RT-qPCR data also showed that CTSZ is dysregulated upon estradiol treatment in all three groups of osteosarcoma and MCF-7 cells as compared to untreated controls of each cell type. CTSZ mRNA expression in PBMC samples was significantly upregulated in osteopenia and postmenopausal osteoporosis in comparison to non-osteoporotic participants' ( $p = 0.0011$ , **Figure 4-14**). Moreover, a significant correlation was detected between CTSZ mRNA expression levels in PBMCs and the participants' T-score ( $p = 0.0002$ ). As evidenced by an AUC value of 0.806, CTSZ was among the strongest diagnostic value through AUC discrimination measurement with sensitivity of 74% and specificity of 90%.

Altogether, it was possible to show that mRNAs/miRNA are associated with the pathogenesis of osteoporosis and may play important roles in the detection and classification of osteoporotic diseases. Some of the identified mRNA/miRNA showed significant difference in distinguishing between non-osteoporotic and osteoporotic female patients. However, it should be pointed out that more studies are necessary to further investigate biological role of the mRNA/miRNA in the bone cells. Similarly, the data here suggest an integral role played by CTSZ in bone resorption. This is accomplished by inducing osteoclastic differentiation of PBMCs, possibly through promoting their transendothelial migration. Hence, manipulating CTSZ-mediated activation of PBMCs may be a promising target for drug development. In order to

achieve that, the biological role of CTSZ in osteosarcoma cells will be investigated as describe in the next chapter.

## **Chapter 5: The Role of Cathepsin Z in Osteosarcoma Cells**

## 5.1. Introduction

Human cathepsin Z (CTSZ) (also known as cathepsin X, CTSX, and cathepsin P, CTSP) is a lysosomal cysteine proteinase and member of the cathepsin protease family (Santamaria et al., 1998), which includes other cysteine cathepsins, namely cathepsins B, C, F, H, K, L, O, S, V, W and Z (Santamaria et al., 1998). CTSZ protein exhibits both carboxy-monopeptidase and carboxy-dipeptidase activities and are found in all animals as well as other organisms (Santamaria et al., 1998). Some of these cathepsins are expressed highly in specific cell types such as CTSK in osteoclasts and CTSS in immune cells (Mohamed and Sloane, 2006). The cysteine cathepsin proteases function predominantly as intracellular endopeptidases in endolysosomal vesicles (Mohamed and Sloane, 2006), where most become activated in the acidic environment of the lysosome (Jedeszko and Sloane, 2004). Cathepsin also function extracellularly through the degradation of the extracellular matrix such as cathepsin K which is the major therapeutic target for osteoporosis (Fonovic and Turk, 2014). The organic bone matrix is degraded by not only cathepsin K, but also by matrix metalloproteinases or other cathepsins (Goto et al., 2003)

The expression of CTSX was found in monocytes, macrophages and dendritic cells (Kos et al., 2005) and in tumour cells of prostate (Nägler et al., 2004), gastric carcinoma (Buhling et al., 2004), and in aging mouse brain cells, glial cells and was also reported in patients with Alzheimer disease (Wendt et al., 2007). CTSX enzyme was found to be secreted from non-hematopoietic bone marrow cells and found predominantly on the surface of primary human osteoblasts (Staudt et al., 2010) but the biological function of CTSZ remains unknown. The expression of cathepsins B, L and X



were downregulated during differentiation of myeloid-derived suppressor cells (MDSCs) from macrophage to osteoclast in mice bearing highly metastatic tumours which might inhibit osteoclastogenesis by decreasing the fusion of osteoclast precursor through cleavage of proteins needed for membrane fusion (Edgington-Mitchell et al., 2015).

In Chapter 4, the expression of cathepsin Z mRNA (CTSZ) was found to be decreased based on the maturity level of the cultured cells. Expression of CTSZ mRNA was also found to be significantly upregulated in PBMC isolated from clinical specimens from osteopaenia and postmenopausal osteoporosis patients compared to specimens from non-osteoporotic control. The expression of CTSZ mRNA was shown to be significantly correlated with T-score of the participants. ROC analysis of the CTSZ were among the highest sensitivity and specificity of the regulated genes tested on the PBMCs of female non-osteoporotic, osteopenia and osteoporosis postmenopausal participants' samples. Thus, it is important to find out the biological role of CTSZ that might be related to the development of osteopenia/osteoporosis. The aim of this chapter is to understand the biological role of CTSZ in which will be approached by:

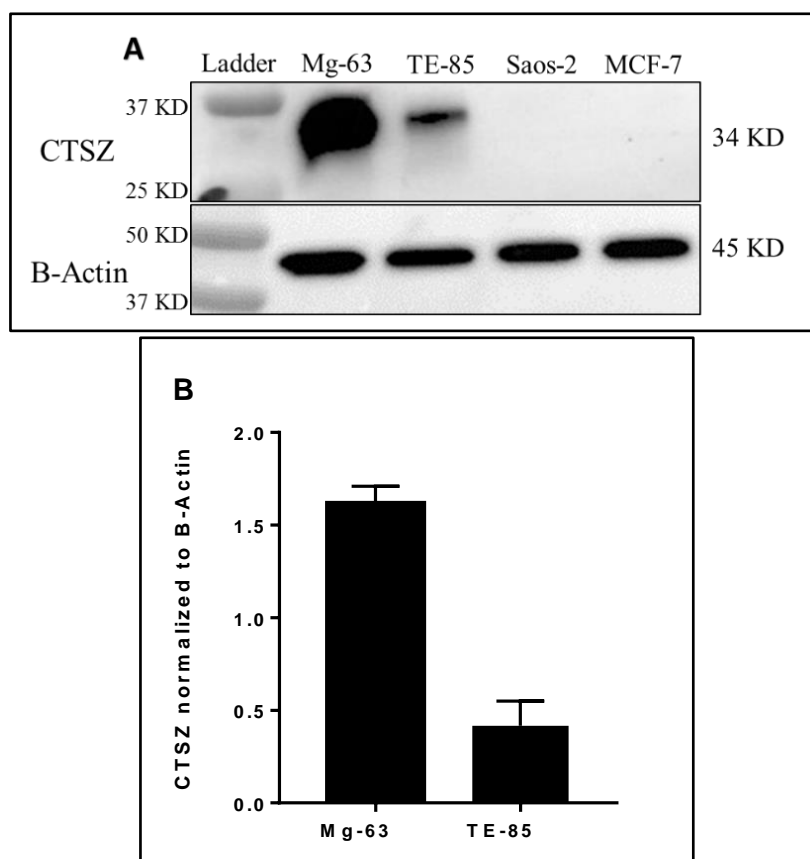
- Finding out whether the cultured osteosarcoma cells produce CTSZ protein.
- Suppressing the expression of CTSZ by RNA interference in cultured osteosarcoma cells.
- Investigating the consequential effect of CTSZ suppression on the proliferation, migration, and invasion of cultured osteosarcoma cells.

## **5.2.Result**

### **5.2.1 Detection of Cathepsin Z protein in the Cultured Osteosarcoma Cells**

In Chapter 4, RT-qPCR results showed that the levels of CTSZ mRNA exhibited an inverse relationship with increasing maturity of osteosarcoma cells. i.e. the levels of CTSZ mRNA were the highest in Mg-63 cells, but lowest in Saos2, while the levels of CTSZ in TE-85 was between that in Mg-63 and Saos2 cells (Chapter 4, Section 0).

In this chapter, initially, the protein levels of CTSZ in the cultured osteosarcoma cells and MCF-7 are determined by western blot using antibodies for CTSZ (R&D System, UK).  $\beta$ -actin antibody was used as a housekeeping control to standardise between samples (Figure 5-1). A single 34 kDa band corresponding to the molecular weight of CTSZ (Pecar Fonovic and Kos, 2015), was detected in the Mg-63 and TE-85 cells, but was undetectable in the Saos-2 and MCF-7 cells. Quantitative analysis showed that the level of CTSZ protein in Mg-63 was 3.8 times higher than that in the TE-85. These results are consistent with the relative levels of CTSZ mRNA in the Mg-63 and TE-85 cell lines (Chapter 4, Section 4.2.1). The detection of low levels of CTSZ mRNA in the Saos-2 and MCF-7 cells, but no protein detection, arises for the better sensitivity of RT-qPCR over western blotting. Mg-63 and TE-85 cells were used for the suppression of CTSZ protein.



**Figure 5-1: Detection of CTSZ protein in the cultured osteosarcoma cells and MCF-7.**

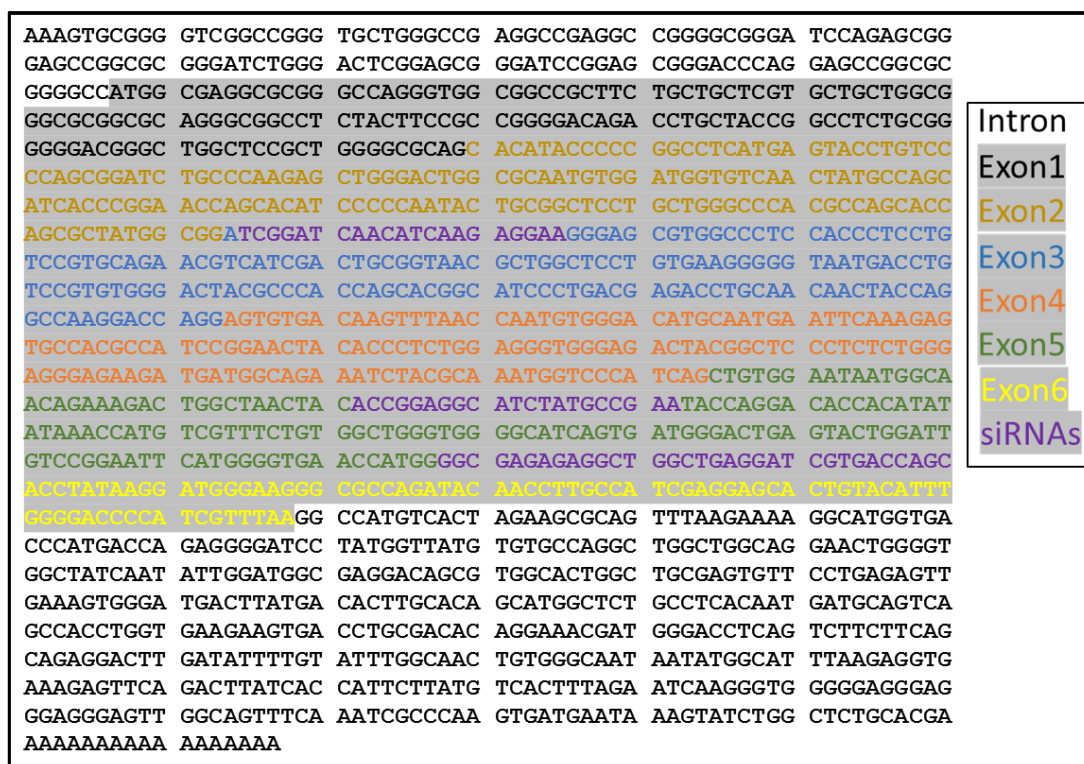
(A) Representative western blot analysis of CTSZ protein levels in cell lines. CTSZ was detected in only the Mg-63 and TE-85 at 34 KDa. However, CTSZ protein was not identified in Saos-2 and MCF-7.  $\beta$ -Actin was used as an internal control. (B) Densitometric analysis of CTSZ protein levels in Mg-63 and TE-85 was calculated. The expression of CTSZ protein in the Mg-63 cells was 3.8 times more than in the TE-8 cells. Each bar represents the mean  $\pm$ SD (n=3).

## **5.2.2 Suppression of Cathepsin Z Expression by RNA Interference**

### **5.2.2.1 SiRNA probes for Cathepsin Z**

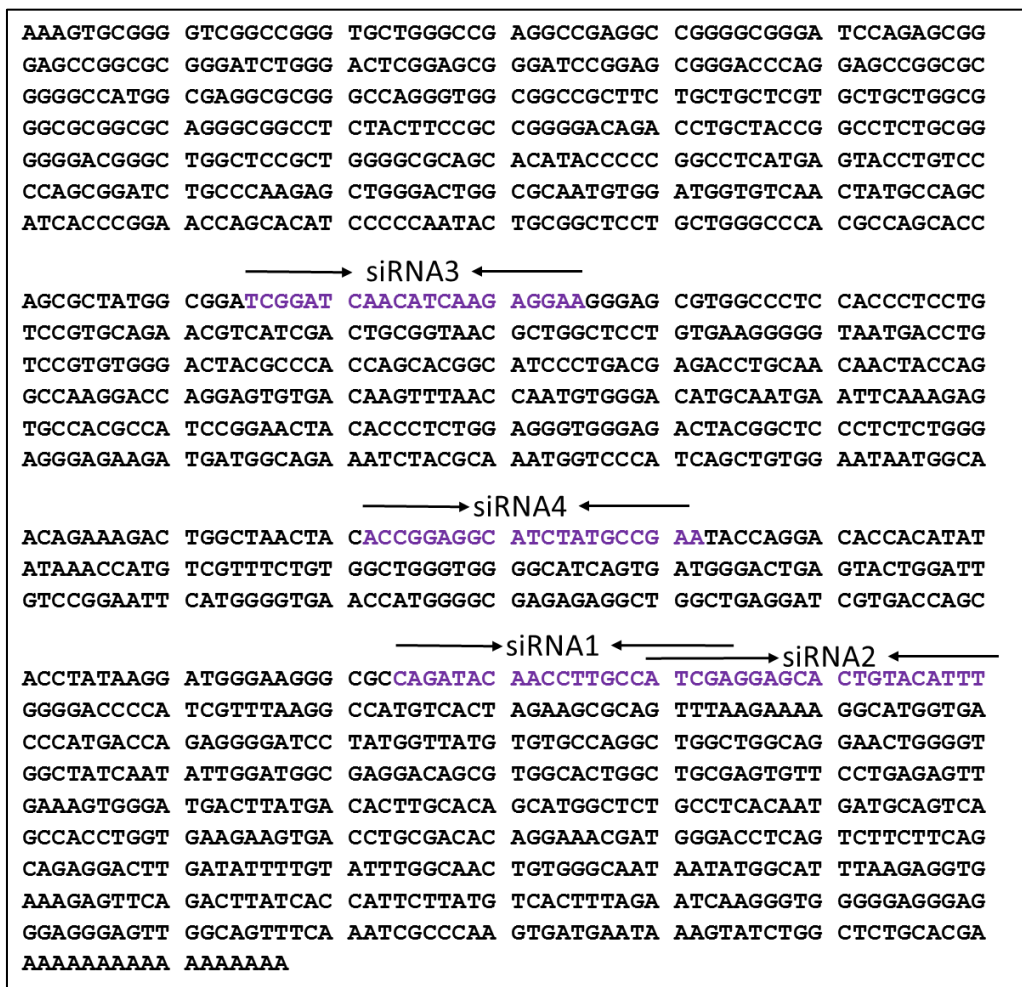
The full-length cDNA sequence of CTSZ and protein sequence are shown in (Figure 5-2). Small-interfering RNA (siRNA) was used to knockdown CTSZ mRNA level in Mg-63 and TE-85 cells in order to assess whether suppression of CTSZ mRNA expression can inhibit CTSZ protein in the Mg-63 and TE-85 cells. 4 target positions on the CTSZ cDNA were selected as highlighted in Figure 5-3 and Table 5-1.

siRNA 1 (CAGATACAACCTTGCCATCGA) located between 984 and 1004, siRNAi 2 (ATCGAGGAGCACTGTACATTT) located between 1000 and 1020, siRNAi 3 (TCGGATCAACATCAAGAGGAA) located between 435 and 455 and lastly siRNA 4 (ACCGGAGGCATCTATGCCGAA) located between 802 and 922. Silencer<sup>®</sup> Negative Control (Ambion, Inc., USA) was used to check for off-target effects or toxicity of transfection reagents or of the siRNA itself.



**Figure 5-2: Full-length mRNA sequence of CTSZ**

The figure showed the full-length cDNA sequence, the accession number is NM\_001336.3. The gray highlighted nucleotide showed the CDS, between 127-1038. 6 exons showed with different colours as well as 4 siRNAs used in this study



**Figure 5-3: Nucleotide sequences of CTSZ mRNA.**

4 siRNA sequences and their positions used in this study to suppress CTSZ expression are coloured purple.

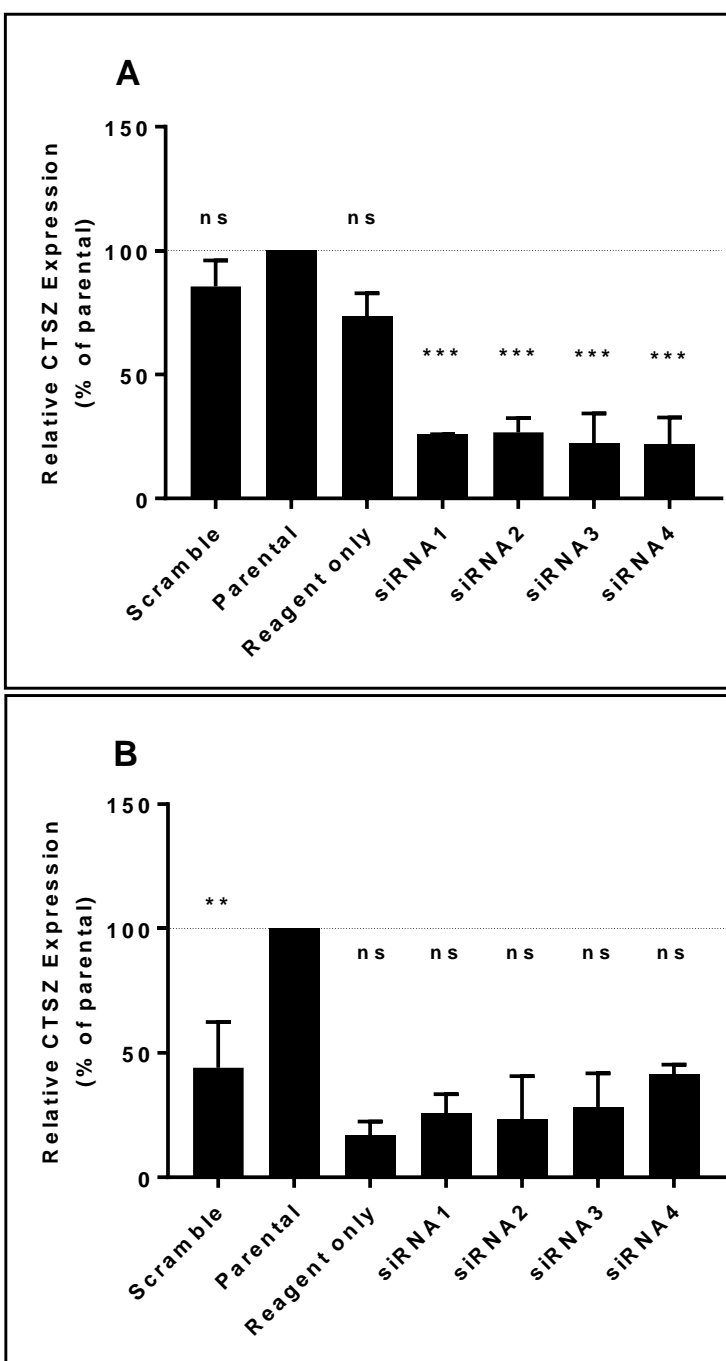
**Table 5-1: List of siRNA primer sequences used**

siRNA	Target Gene	Target sequence	Company	Catalog no.
siRNA 1	CTSZ	CAGATACAACCTTGCCATCGA	Qiagen	SI03064495
siRNA 2		ATCGAGGAGCACTGTACATTT	Qiagen	SI03048087
siRNA 3		TCGGATCAACATCAAGAGGAA	Qiagen	SI00025536
siRNA 4		ACCGGAGGCATCTATGCCGAA	Qiagen	SI00025529

### 5.2.2.2 Test Time Course of siRNA Transfection on Osteosarcoma Cells

Initial experiments were directed towards finding out whether incubation of the cells with transfection complexes for 24 or 48 h was necessary to achieve a reduction in CTSZ mRNA levels. Mg-63 cells were used for these experiments (Figure 5-4 and Table 5-2). The expression of CTSZ on negative control, and siRNA-treated Mg-63 cells were compared with the expression of CTSZ on scrambled siRNA using one-way ANOVA. Quantitative analysis showed that the levels of CTSZ mRNA in scrambled control and Mg-63 treated with transfection reagent alone were similar to that in the parental Mg-63 cells. For cells incubated with transfection reagent for only 24 h (Figure 5-4A), there was an insignificant decrease in expression of CTSZ mRNA when compare to the scrambled siRNA ( $p = 0.795$ ). However, for cells treated with CTSZ siRNA oligonucleotides the expression of CTSZ mRNA was decreased by around 60 % when compared to the scramble siRNA ( $p = 0.0019, 0.002, 0.0013$  and  $0.0012$  for siRNA 1, 2, 3 and 4 respectively).

After 48 h (Figure 5-4, B), the levels of CTSZ mRNA transfected with CTSZ-siRNA were significantly reduced relative to the parental control ( $p = 0.008$ ), but were not significantly different to the scrambled control ( $p > 0.05$ ) and the transfection reagents only ( $p > 0.05$ ), which were similarly reduced. This shows that after 48 h, the transfection reagent itself is causing a reduction in the expression of CTSZ mRNA and not the siRNA. Thus, 24 h was used in future siRNA experiments due to toxicity of transfection reagent after 48 h.



**Figure 5-4: RT-qPCR results for Mg-63 cells treated with SiRNAs for 24 and 48 h.**

CTS<sub>Z</sub> silencer and negative control were added to the confluent cells after which, cells were harvested after 24 (A) or 48 h (B). Relative expression of CTS<sub>Z</sub> mRNA was determined by  $2^{-\Delta CT}$  method, normalized to GAPDH. Each bar represents the mean  $\pm$ SD (n=2). Negative controls and infected Mg-63 by CTS<sub>Z</sub> siRNAs were compared to the parental Mg-63 which was set as 100 using one-way ANOVA.



**Table 5-2: RT-qPCR results for optimization the time course of the transfection reagent and siRNAs of CTSZ in Mg-63 for 24 and 48 h.**

Incubation time	Dunnett's multiple comparisons test	Mean Difference to the scrambled siRNA (%)	P Value
24 h	Parental vs. Scramble	14.42	0.3418
	Reagent only vs. Scramble	-12.08	0.7955
	siRNA1 vs. Scramble	-57.04	0.0019
	siRNA2 vs. Scramble	-56.23	0.0020
	siRNA3 vs. Scramble	-60.81	0.0013
	siRNA4 vs. Scramble	-61.05	0.0012
48 h	Parental vs. Scramble	55.99	0.0078
	Reagent only vs. Scramble	-27.08	0.1833
	siRNA1 vs. Scramble	-18.33	0.4717
	siRNA2 vs. Scramble	-20.72	0.3703
	siRNA3 vs. Scramble	-15.66	0.6025
	siRNA4 vs. Scramble	2.6	0.9997

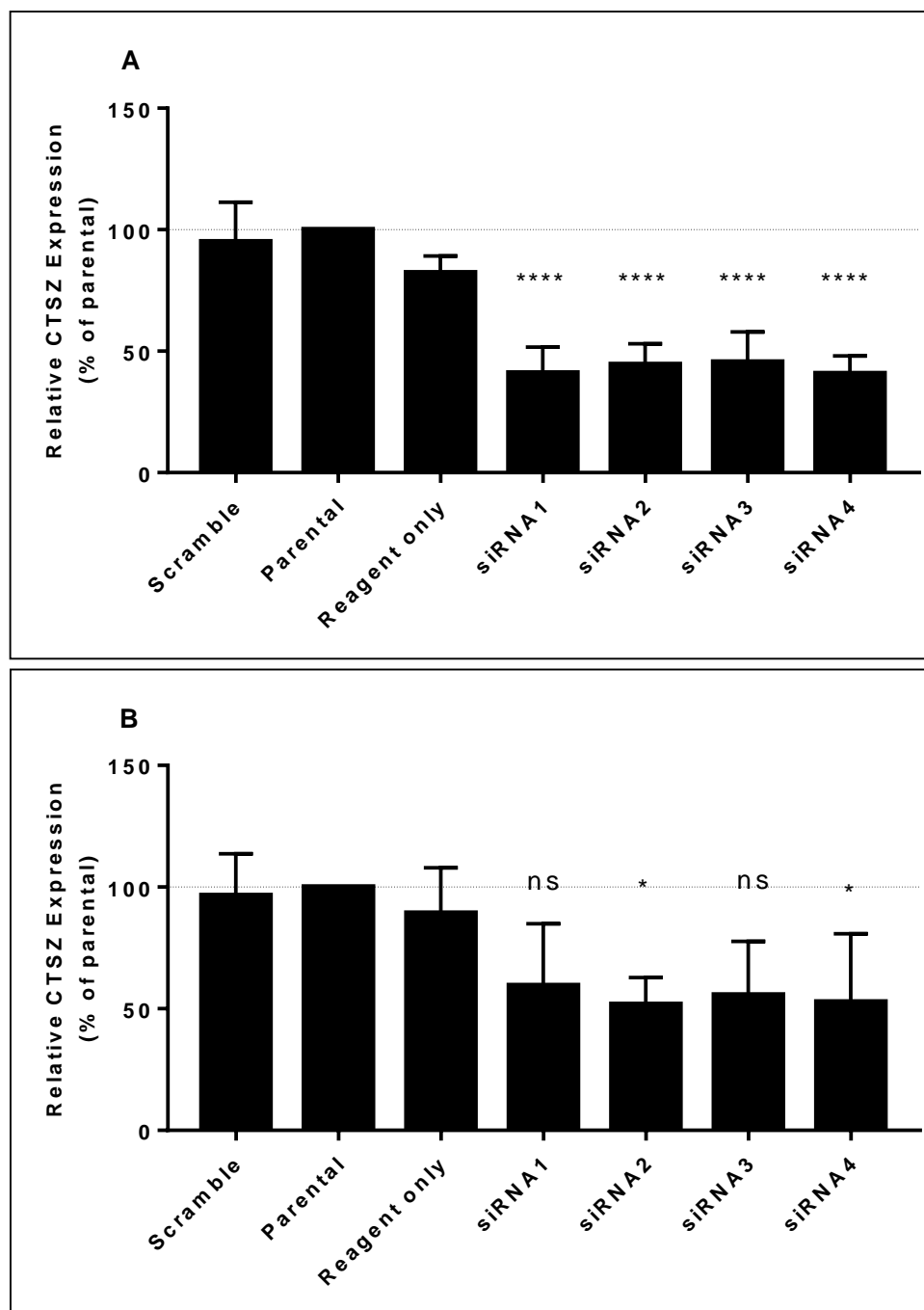
Cells infected with negative control and silencer CTSZ siRNAs were compared to the parental cells using one-way ANOVA, (Dunnett's multiple comparisons test). The mean difference was calculated by the percentage of decreased expression to the parental.

### **5.2.2.3 Effect of siRNA on the Level of CTSZ mRNA in Osteosarcoma Cell Line Mg-63 and TE-85**

To assess the extent of suppression of CTSZ mRNA in Mg-63 and TE-85 cells, mRNA was isolated from parental, transfected with scrambled siRNA and CTSZ-siRNA transfected Mg-63 and TE-85 cells, followed by reverse transcription. The relative levels of CTSZ mRNAs were analysed by quantitative RT-qPCR using GAPDH as a housekeeping control, Figure 5-5 (A) for Mg-63 and Figure 5-5 (B) for TE-85.

The results showed that the levels of CTSZ mRNA in Mg-63 cells transfected with scrambled control siRNA and treated with transfection reagent only were similar to that in the parental untreated Mg-63. However, the level of CTSZ mRNA was significantly reduced compared to scrambled control in all CTSZ-siRNAs ( $p < 0.0001$ ) 54, 50.5, 49.5 and 54.25 % in cells transfected with CTSZ-siRNA 1, 2, 3, or 4, respectively.

Additionally, the level of CTSZ mRNA expression in TE-85 was significantly decreased in cells transfected with siRNAs 2 and 4 by 44.8 and 43.8% relative to the scrambled control cells ( $p = 0.02$  and  $0.022$ , respectively), but not significantly by 37 and 40.9 % for SiRNAs 1 and 3 compared to the scrambled control ( $p = 0.055$ , and  $0.06$ , respectively).



**Figure 5-5: The effect of CTSZ mRNA suppression in Mg-63 and TE-85 cells using CTSZ-siRNAs**

CTSZ silencer and control SiRNAs were added to confluent cells, after which the cells were harvested after 24 h. Data represent the relative expression of CTSZ determined by the relative Ct method normalized to GAPDH for Mg-63 (A) and TE-85 (B). Each bar represents the mean and  $\pm$ SD (n=3).

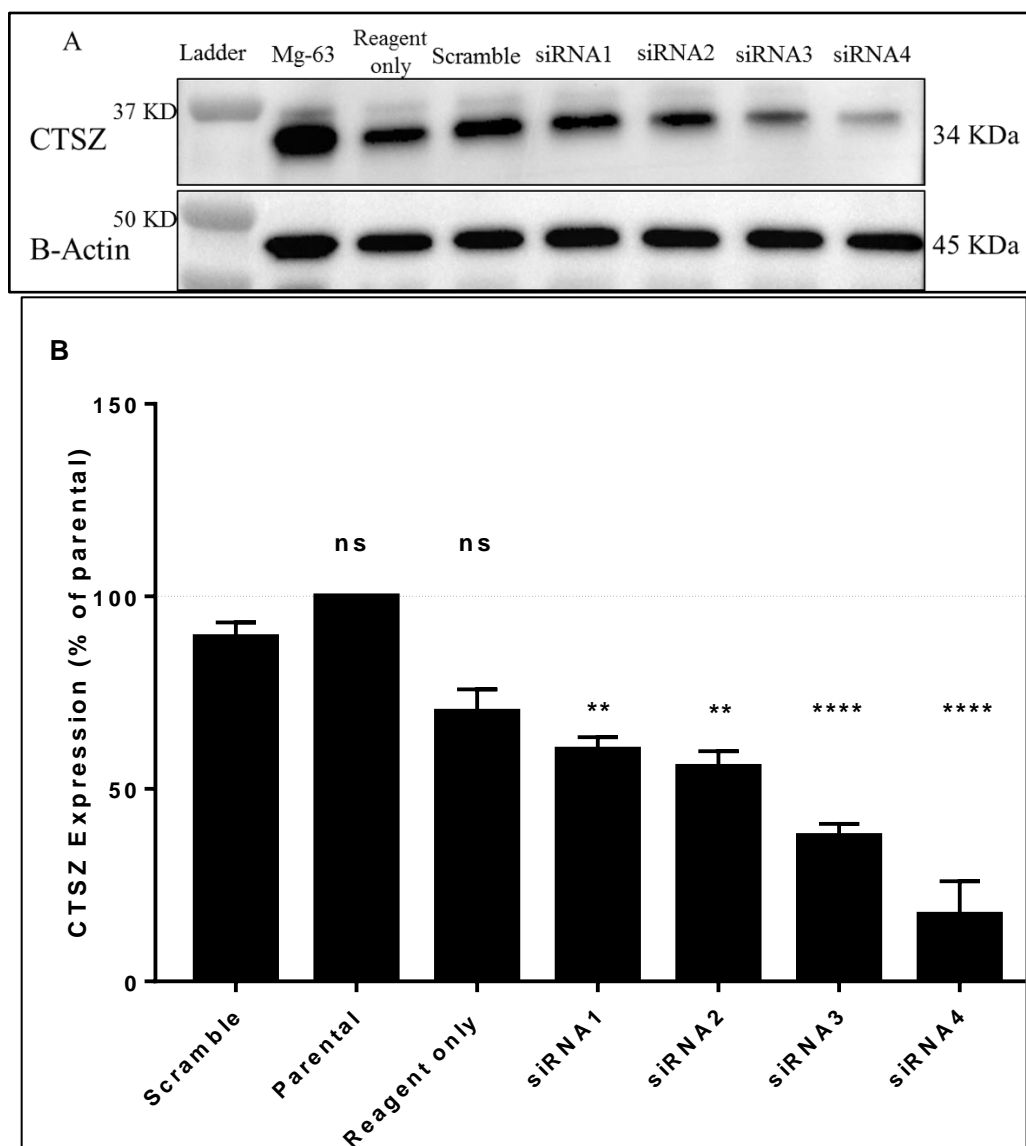
#### **5.2.2.4 Effect of siRNA on the Level of CTSZ Protein in Osteosarcoma Cell Lines Mg-63 and TE-85.**

Since the CTSZ protein was only found in Mg-63 and TE-85 cells, the suppression of CTSZ protein was then measured in these cells. Western blot analysis of the knockdown CTSZ protein expression in Mg-63 cells is shown in Figure 5-6 and Table 5-3, which summarizes the percentage changed statistically with the expression of CTSZ protein in the parental of Mg-63 set as 100.

A single CTSZ band of 34 kDa was detected in parental Mg-63, negative controls, and CTSZ-siRNA transfected cells. The levels of CTSZ protein expression in CTSZ-siRNAs transfected Mg-63 cells were reduced compared to the parental control cells (Figure 5-6 A). Densitometric analysis showed that the level of CTSZ protein in Mg-63 cells treated with CTSZ-siRNA 1, 2, 3 and 4 was significantly reduced by 29 (p = 0.0034), 34 (p = 0.001), 52 (p < 0.0001) and 72 % (p < 0.0001), respectively compared to the scrambled control (one-way ANOVA, Dunnett's multiple comparisons test). The negative controls using reagent-only showed a small non-significant 8% decrease in CTSZ protein level (p = 0.054) compared to scrambled control.

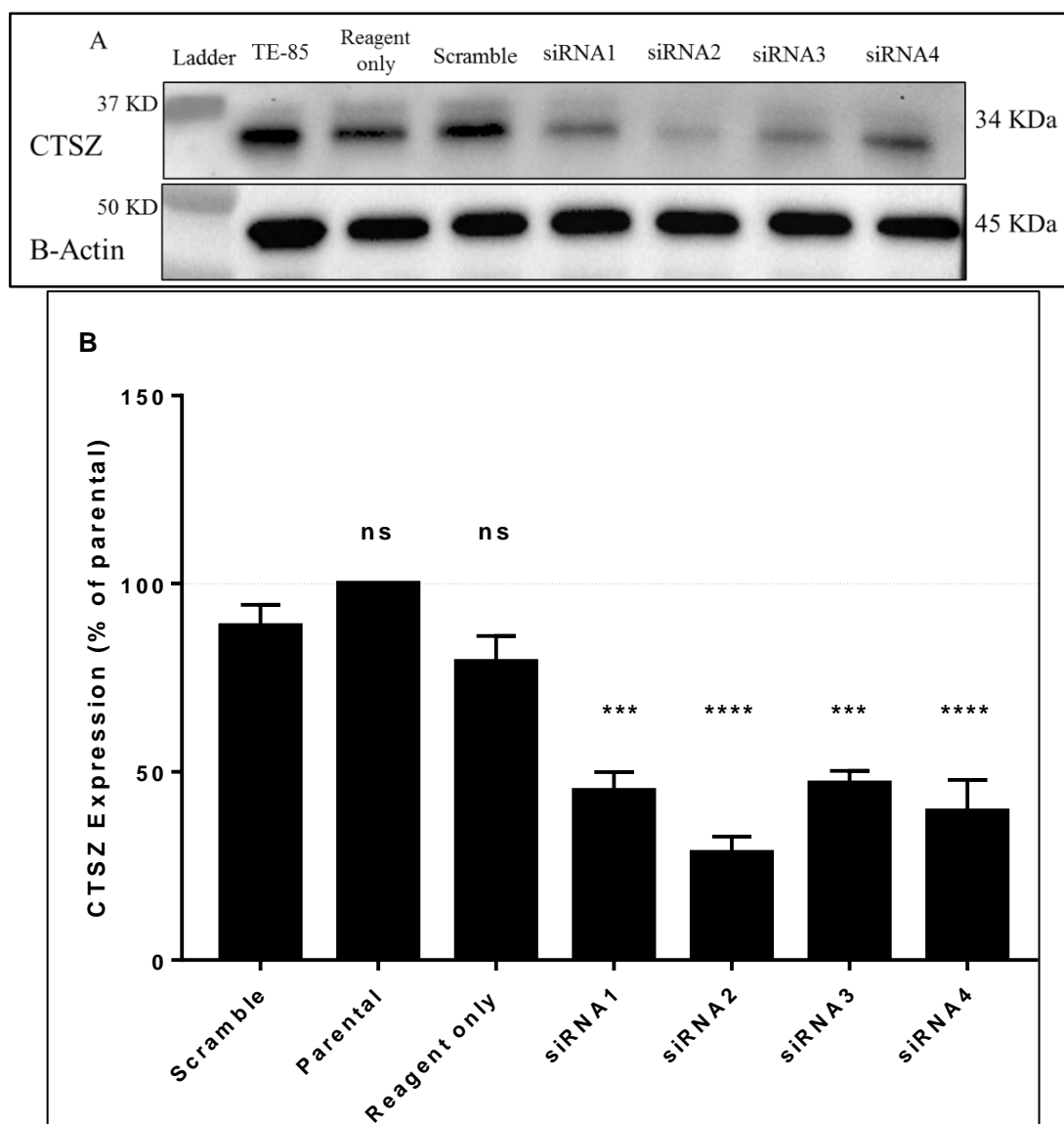
The levels of CTSZ protein in scrambled control and TE-85 treated with transfection reagent alone were similar to that in the parental TE-85 cells, (Figure 5-7). The relative levels of CTSZ protein in TE-85 cells transfected with CTSZ-siRNA 2, 4, 1 and 3 were decreased by 60.2, 49.2, 43.8 and 41.8 % respectively compared to that in the scrambled control. The most efficient suppression was achieved in TE-85 by siRNA-2, up to 60 % (p = 0.0001) compared to scrambled control. Thus, Mg-63-CTSZ siRNA4

and TE-85-CTSΖ-siRNA 2 showing the highest effect of the CTSΖ suppression were used for the function tests.



**Figure 5-6: The effect of CTSΖ-siRNA on the levels of CTSΖ protein in Mg-63 cells.**

(A) Representative western blot analysis of CTSΖ levels in Mg-63 transfected with non-small interfering control or small interfering CTSΖ. Cells were harvested after 24 h of treatment.  $\beta$ -Actin was used as an internal control. (B) Densitometric analysis of CTSΖ protein levels in Parental or treated with CTSΖ-siRNA. All experiments were repeated three times, and data are expressed as mean  $\pm$  SD (n=3). ns= not significant, p value \*\* < 0.01, \*\*\*\* < 0.0001, = significant



**Figure 5-7: CTSZ protein expression was decreased by siRNA knockdown in TE-85 cells.**

CTSZ protein expression was evaluated by (A) western blotting with lysates from cells that were treated with a 24 h transfection with siRNAs or negative control. (B) The relative intensities of the bands on the western blot were analyzed using Image lab Software. Columns indicate the ratio of CTSZ intensity to  $\beta$ -Actin intensity that was measured in three independent experiments. ns= not significant, p value \*\*\* < 0.001, \*\*\*\* < 0.0001, = significant

**Table 5-3: Suppression of CTSZ protein expression by siRNA knockdown in Mg-63 and TE-85 cells.**

	<b>Dunnett's multiple comparisons test</b>	<b>Mean Difference to the Scramble cells (%)</b>	<b>P Value</b>
<b>Mg-63</b>	Parental vs. Scramble	10.5	0.4949
	Reagent only vs. Scramble	-20.4	0.0502
	siRNA1 vs. Scramble	-29.3	0.0046
	siRNA2 vs. Scramble	-33.7	0.0014
	siRNA3 vs. Scramble	-51.7	0.0001
	siRNA4 vs. Scramble	-72.1	0.0001
<b>TE-85</b>	Parental vs. Scramble	11.2	0.5303
	Reagent only vs. Scramble	-5.3	0.9578
	siRNA1 vs. Scramble	-43.8	0.0003
	siRNA2 vs. Scramble	-60.2	0.0001
	siRNA3 vs. Scramble	-41.8	0.0005
	siRNA4 vs. Scramble	-49.2	0.0001

Each of cells transfected with negative control and silencer CTSZ siRNAs were compared to the scramble cells using one-way ANOVA (Dunnett's multiple comparisons test). The expression of CTSZ protein in untreated Mg-63 and TE-85 was set as 100. The mean difference was calculated by the percentage of decreased expression compared to the scrambled control.

### **5.2.3 Silencing of Cathepsin Z Inhibit the proliferation, Invasion, and Migration of Mg-63 and TE-85**

#### **5.2.3.1 Effect of CTSZ-siRNAs on the Proliferation of Mg-63 and TE-85 Cells**

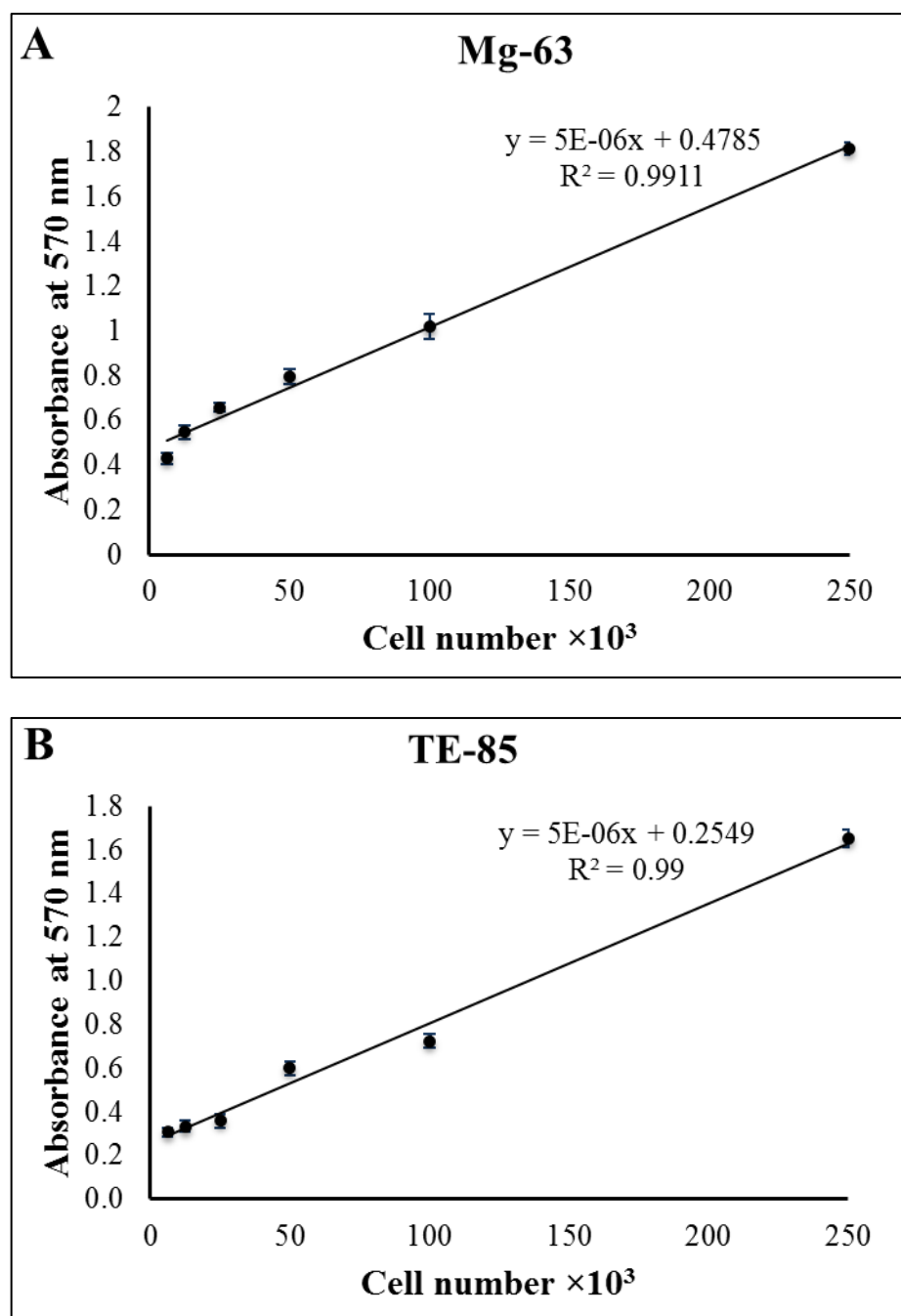
The effect of CTSZ suppression on the proliferation of Mg-63 and TE-85 was assessed by MTT assay. A standard curve for each cell line was generated to correlate the optical density of formazan with the cell number as shown in Figure 5-8. Linear regression indicated a linear correlation ( $R^2$  for Mg-63 and TE-85 cells both 0.99) between absorbance signal, at 570 nm, and cell number ranging from  $6.25 \times 10^3$  to  $2.5 \times 10^5$  cells in the volume of the assay. Thus, the values of absorbance at 570 nm showed a strong correlation with the number of cells seeded from that range. Then, 5000 cells from parental, control and siRNAs transfected of Mg-63 and TE-85 cell line were seeded and the absorbance was measured over a 6-day period. The optical density values obtained from the plate reader were converted to cell numbers, which were extrapolated from the standard curve.

Overall, growth rate of parental cells and transfectants displayed a very similar growth pattern (Figure 5-9). Statistical analysis using two-way ANOVA (Dunnett's multiple comparisons test) (Table 5-4) showed no significant difference at any time point, including day 6, between parental, reagent only and scrambled siRNA transfectants control for both Mg-63 and TE-85 ( $P > 0.05$ ). By the 4<sup>th</sup> day, cell numbers were significantly decreased by 44 % in Mg-63 treated with CTSZ-siRNA4 ( $P = 0.0034$ ). On the 5<sup>th</sup> day, the cell number was significantly reduced for those cells treated with CTSZ-siRNA 4, 2, 3 and 1 by 36, 33, 30 and 23 %, respectively, compared to the



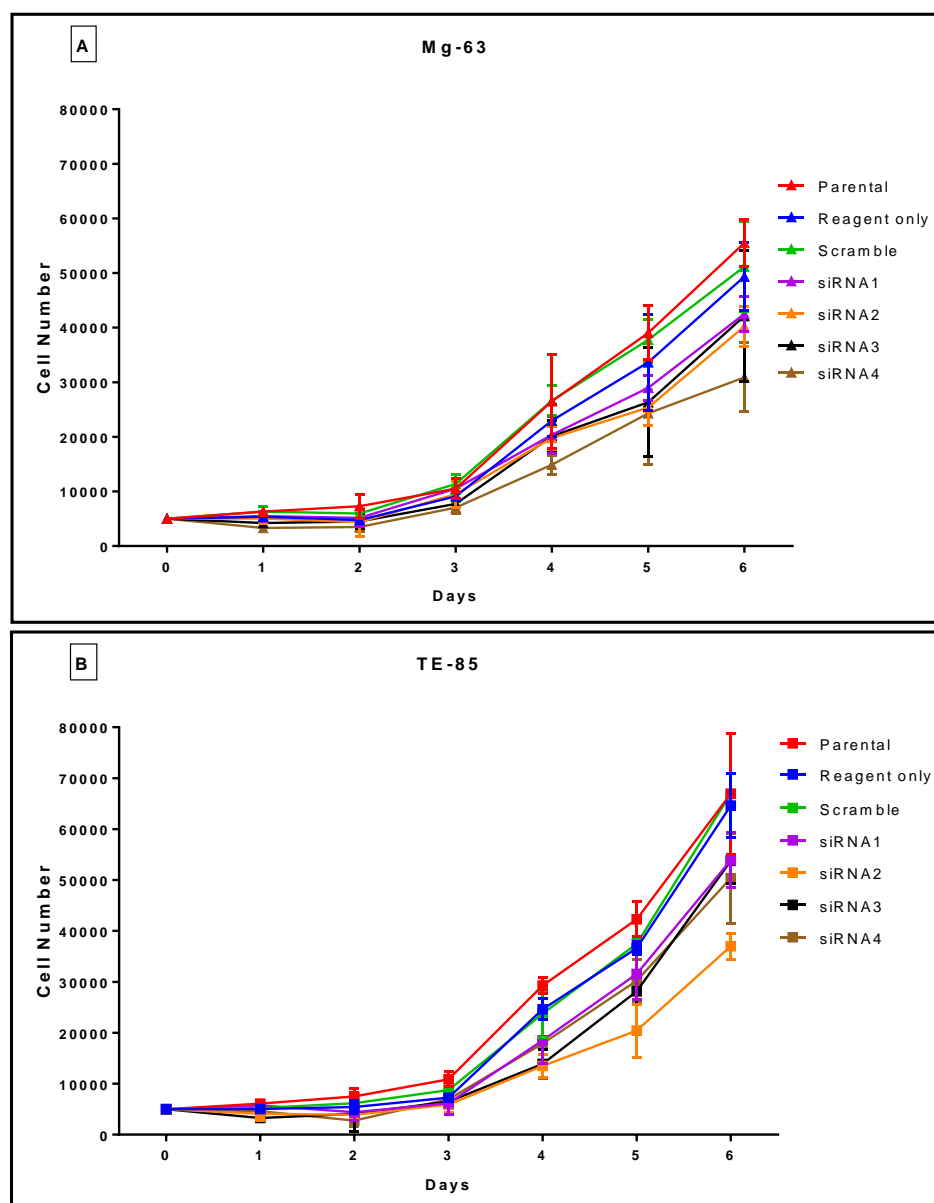
scrambled siRNA control. By day 6, the Mg-64 cells expressing the 4 siRNAs all showed a significant decrease in the cell number (Table 5-4) with Mg-64-CTSZ-siRNA4 showing the greatest inhibitory effect on growth rate of 40%. SiRNA 4 was the most effective siRNA at reducing CTSZ levels (72% reduction).

Transfectant TE-85 with all CTSZ-siRNAs showed a significant decrease in cell number starting from day 4, and by day 6 cell numbers were decreased by 45, 25, 20% and 20% for CTSZ-siRNA 2, 4, 1 and 3 transfected cells, respectively ( $p = 0.0001$ ). In this case, siRNA 2 was also the most effective at reducing the growth rate. In TE-85 cells, siRNA2 was the most effective at reducing the level of CTSZ protein, thus in both Mg-63 and TE-85 cells, reduction of CTSZ protein is associated with a significant reduction in growth rate. This interpretation is strengthened by the observation that plots of % reduction in CTSZ protein and reduction in growth rate showed a linear relationship, with  $R^2$  values for Mg-63 and TE-85 of 0.78 and 0.96, respectively (Table 5-4).



**Figure 5-8: The relationship between colour development and cell number in the MTT assay of cell growth of Mg-63 and TE-85 cells.**

Using linearity, standard curves were established for Mg-63 cells (A) and TE-85 cells (B) as described in Materials and Methods. The curve equation and regression of each standard curve is presented on the diagrams. Each point represents mean  $\pm$ SD (n=3).



**Figure 5-9: the impact of CTSZ suppression on the proliferation rate of transfectant cells.**

The growth rate of untransfected parental, scrambled and CTSZ-siRNAs transfectants of Mg-63 (A) and TE-85 (B) cells during a 6- day experimental period and samples subjected to MTT assay. The cell count for each sample was determined from a calibration curve. Data showed as mean  $\pm$ SD (n=3).

**Table 5-4: The effect of suppressed CTSZ on the growth rate of Mg-63 and TE-85 at the end points of proliferation assay**

	<b>Mg-63</b>		<b>TE-85</b>	
<b>Dunnett's multiple comparisons test</b>	<b>Changes to parental (%)</b>	<b>P value</b>	<b>Changes to parental (%)</b>	<b>P value</b>
<b>Parental vs. Scrambled</b>	-8	0.6	0	0.99
<b>Reagent only vs. Scrambled</b>	4	0.98	3	0.94
<b>siRNA1 vs. Scrambled</b>	12	0.04	20	0.0005
<b>siRNA2 vs. Scrambled</b>	21	0.007	45	0.0001
<b>siRNA3 vs. Scrambled</b>	18	0.04	20	0.0004
<b>siRNA4 vs. Scrambled</b>	40	0.0001	25	0.0001

The percentage of cell counts on day 6 is shown compared to the scrambled control. P values were obtained by two-way ANOVA (Dunnett's multiple comparisons test).

### 5.2.3.2 Effect of CTSZ-siRNAs on the Invasion of Mg-63 and TE-85 Cells through Matrigel.

The impact of CTSZ -siRNAs on invasiveness of osteosarcoma cells Mg-63 and TE-85 was assessed by a transwell chemotactic invasion assay using a BD BioCoat™ Matrigel™. The invaded cells were counted in four random fields using light microscope at  $400\times$  magnification as shown in Figure 5-10. One-way ANOVA (with post hoc Dunnett's multiple comparisons test) was used to compare the mean of the transfectant cells with the scrambled control while parental cells were set as 100 as shown in Figure 5-11 and Table 5-5.

The invasion of parental Mg-63 cells and transfection reagent-only cells have similar penetration rates ( $P = 0.81$  and  $0.44$ , respectively compared to scramble). Cell transfected with CTSZ-siRNA showed a decreased rate of invasiveness compare to the scrambled siRNA ( $P = 0.0001$ ). The maximum depletion of invasion among the CTSZ-siRNAs was by siRNA-4 at  $69.9\%$  ( $p = 0.0001$ ). The second highest reduction was shown in CTSZ-siRNA-2 with  $63.6\%$  ( $p = 0.0001$ ). While CTSZ-siRNA-3 and 1 showed lower but still significant reductions in the rates of invasion of  $58.6$  and  $57.6\%$  ( $p$  value of both =  $0.0001$ ).

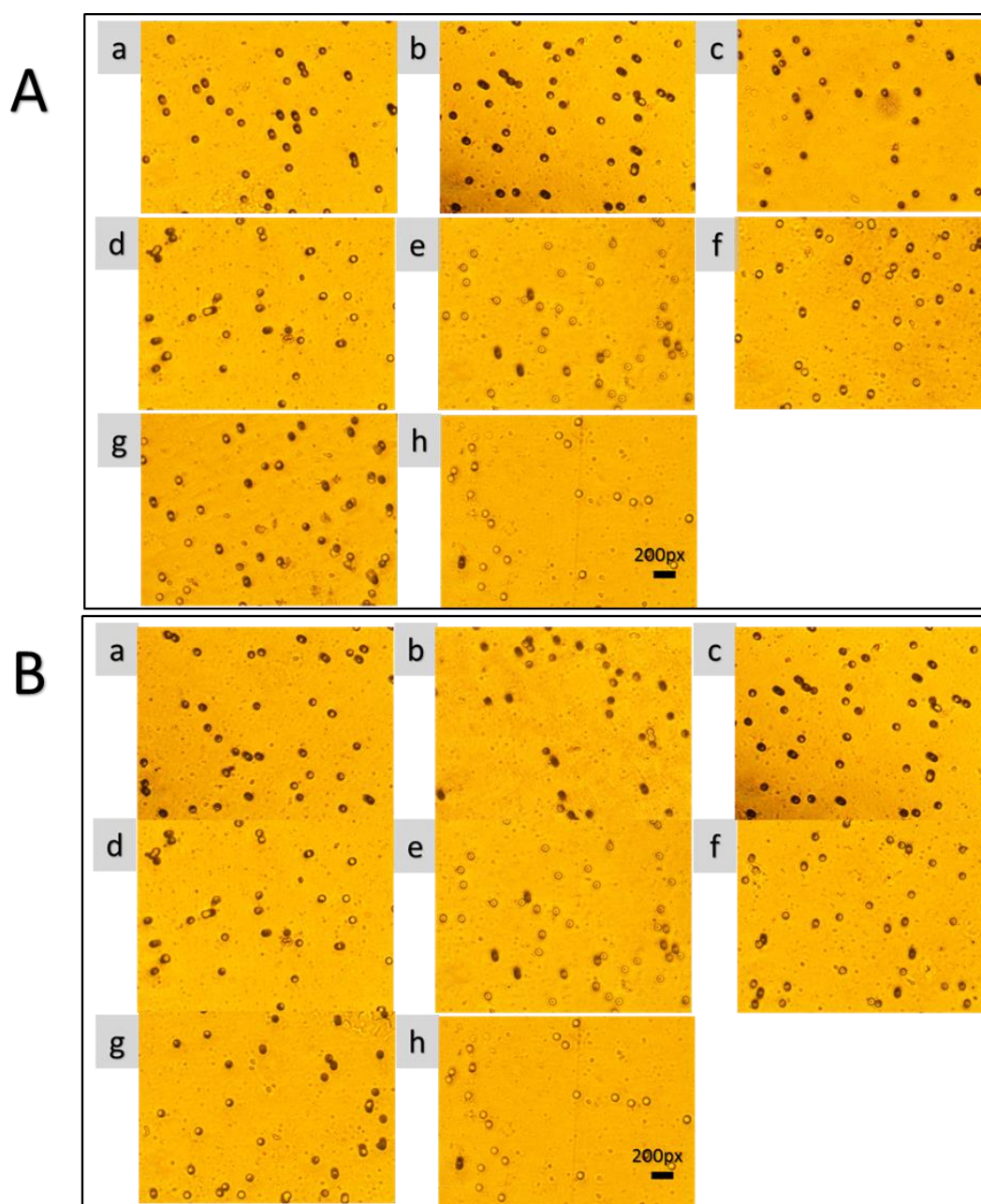
The penetration rate of parental TE-85 cells and transfection reagent-only cells showed the same rate of invasiveness once compared to the scrambled siRNA ( $P = 0.998$  and  $0.94$ , respectively). TE-85 transfected with CTSZ-siRNAs showed a significant reduction in the rate of invasiveness compared to that in the scrambled. The highest reduction was revealed to be in CTSZ-siRNA-2 by  $65.1\%$  ( $P = 0.0001$ ). The second highest reduction was shown in CTSZ-siRNA-3 by  $43.2\%$  ( $P = 0.0001$ ), While CTSZ-

siRNA-1 and 4 showed significantly lower rates of the invasion of 26 and 21 %, respectively,  $P= 0.0014$  and  $0.011$ , respectively. Overall, the invasion of cells was decreased when the level of CTSZ was reduced.

**Table 5-5: The impact of CTSZ silencing on invasion rates of Mg-63 and TE-85 cells.**

Cell line	Dunnett's multiple comparisons test	The difference rate of the invasion mean compared to scramble	P Value
<b>Mg-63</b>	Parental vs. Scramble	4.91	0.8126
	Reagent only vs. Scramble	-7.388	0.4395
	siRNA1 vs. Scramble	-57.64	0.0001
	siRNA2 vs. Scramble	-63.55	0.0001
	siRNA3 vs. Scramble	-58.62	0.0001
	siRNA4 vs. Scramble	-69.95	0.0001
	negative vs. Scramble	-90.15	0.0001
<b>TE-85</b>	Parental vs. Scramble	2.617	0.9977
	Reagent only vs. Scramble	-5.183	0.9410
	siRNA1 vs. Scramble	-26.03	0.0014
	siRNA2 vs. Scramble	-65.08	0.0001
	siRNA3 vs. Scramble	-43.22	0.0001
	siRNA4 vs. Scramble	-21.35	0.0110
	negative vs. Scramble	-90.08	0.0001

The mean difference is the percentage of cells invaded compared to the parental cells and analysed statistically using one-way ANOVA, (Dunnett's multiple comparisons test). Results were obtained from two separate experiments.

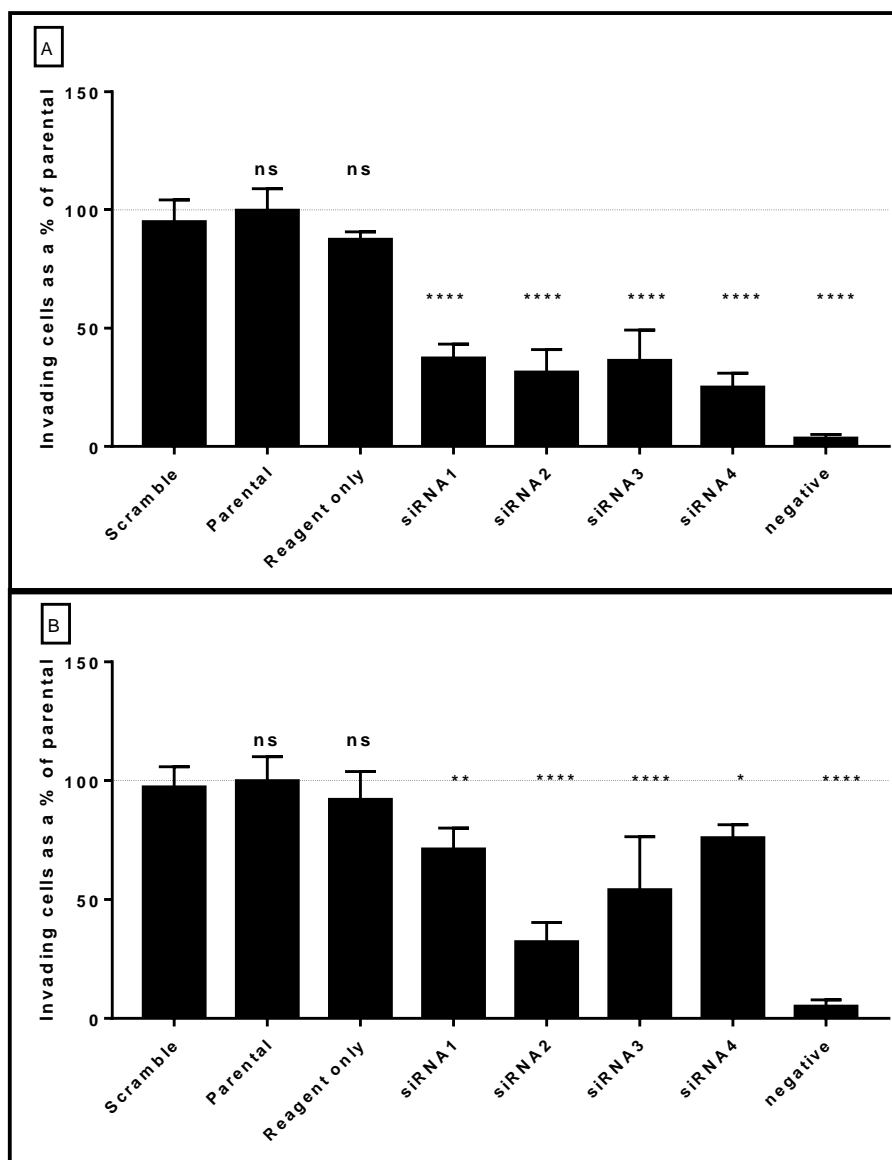


**Figure 5-10: Suppression of Cathepsin Z inhibits the invasion of (A) Mg-63 and (B) TE-85 cell line.**

Cell invasion were tested using Matrigel™ Invasion Chambers.

a,b,c,d,e,f,g and h represent Parental cells, Scramble, Reagent only, siRNA1, siRNA2, siRNA3, siRNA4 and negative control respectively.

Cells in siCTSZ group showed a much lower penetration rate than cells in Parental or the scrambled control. Cells were counted with a microscope at magnification X400 from 4 different fields in 2 experiments.



**Figure 5-11: Suppression of Cathepsin Z partially inhibits the invasion of (A) Mg-63 and (B) TE-85 cell lines.**

Cell invasion were tested using Matrigel™ Invasion Chambers. Cells in the siCTSZ group showed a much lower penetration rate than cell in parental or control cells and importantly the scrambled control. Bars represent mean and  $\pm$  SD of 4 high power fields (n=2). Serum-free medium in the lower chamber was set up as negative control for each cell line.



### **5.2.3.3 Effect of CTSZ-SiRNAs on the Migration of Mg-63 and TE-85**

#### **Cells.**

To evaluate the functional effect of CTSZ knockdown on the migration of the cells, wound healing assays were performed. The migration of the cells, on the two edges of the scratch, were monitored for 72 h or until cell-cell contact was reached the

scratch wound was closed as shown in Figure 5-12 and

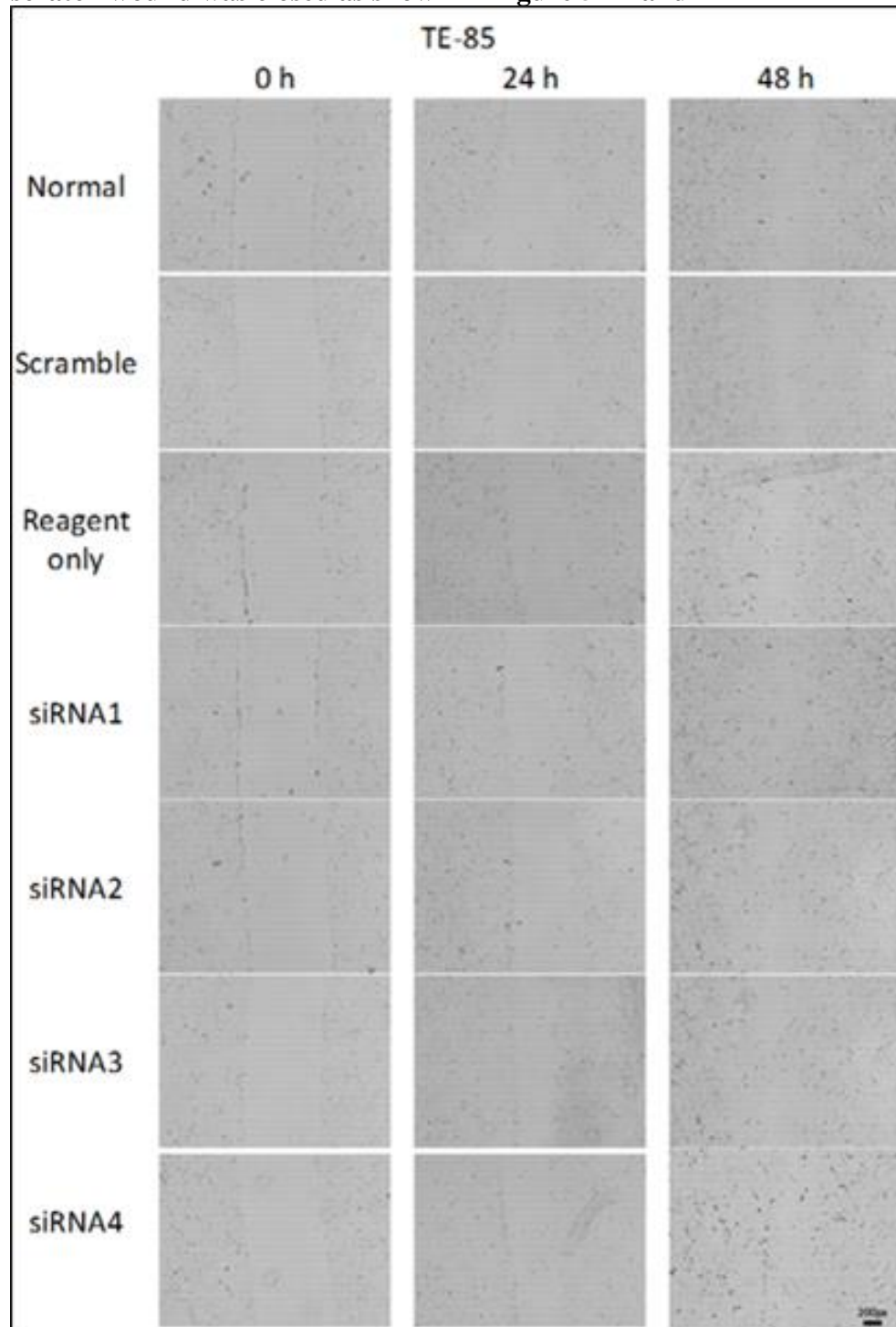
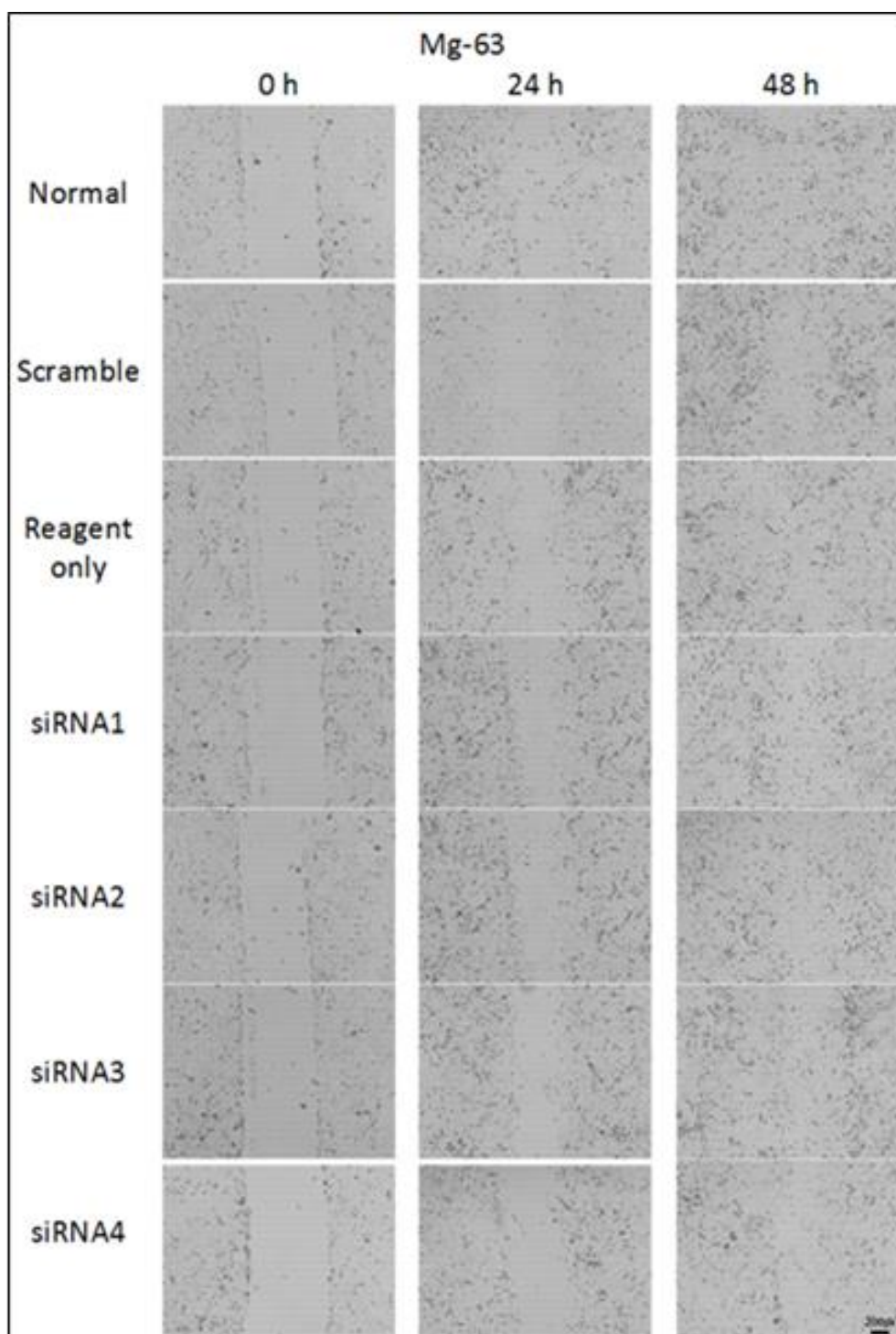


Figure 5-13. Two-way ANOVA (Dunnett's multiple comparisons test) was used to assess the migration rate for the three-time points, 0, 24 and 48 h, and compared to the mean of the scrambled control width as shown in Figure 5-14 and Table 5-6.

The results showed that transfectants with any of the four CTSZ-siRNAs did not exhibit any significant difference in wound closure when compared to the scrambled control in 24 h either in Mg-63 or TE-85 cells (All  $P > 0.05$ ).

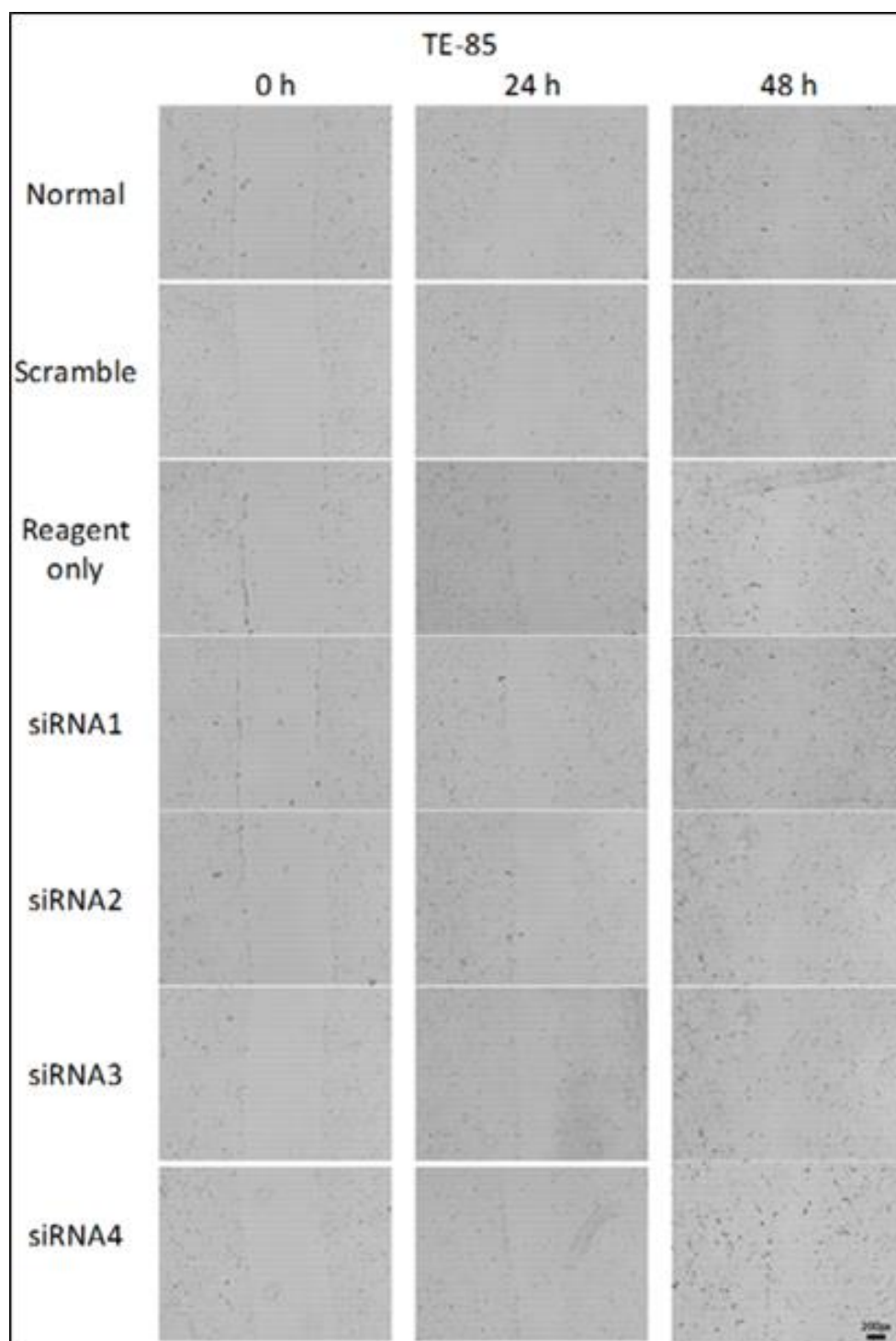
On the other hand, after 48 h, silencing of CTSZ in Mg-63 with any of the four siRNAs showed significant reductions in the rate of migration over 48 h compared with the scrambled siRNA control. The migration of cells with siRNA-4, which had the lowest level of CTSZ, displayed the highest unclosed width of 82 % ( $p < 0.0001$ ) compared to the scrambled control. It also showed that the migration of cells transfected with CTSZ-siRNA-2 has the second highest unclosed width of 78 % ( $p < 0.0001$ ). 63 and 42.3% of the migration rate were shown in Mg-63 with siRNA1 and 3 with  $p$  values of 0.0001 and 0.0017, respectively.

TE-85 cells transfected with CTSZ-siRNAs exhibited a significantly reduced migration rate over 48 h compared with scrambled control. The highest significant migration rate was in cells suppressed by CTSZ-siRNA-2 by 23.9 % ( $p = 0.0015$ ). Furthermore, the migration rate showed a significant decreased in TE-85 with siRNA3, 1 and 4 ( $p = 0.0047$ , 0.011 and 0.0135, respectively). Overall, the migration of cells was decreased with reduced level of CTSZ.



**Figure 5-12: In vitro scratch wound healing assay of Mg-63 cells after silencing CTSZ expression**

Representative images of wound healing with control and different transfectants in 0, 24, 48 and 72 h after the transfection of siRNA Mg-63. Cells were wounded and monitored with microscope every 24 h. Images were captured using a 4x objective lens, were photographed with an Touptek ToupView and the width of the wound between the two edges was measured in triplicate using Image J software.



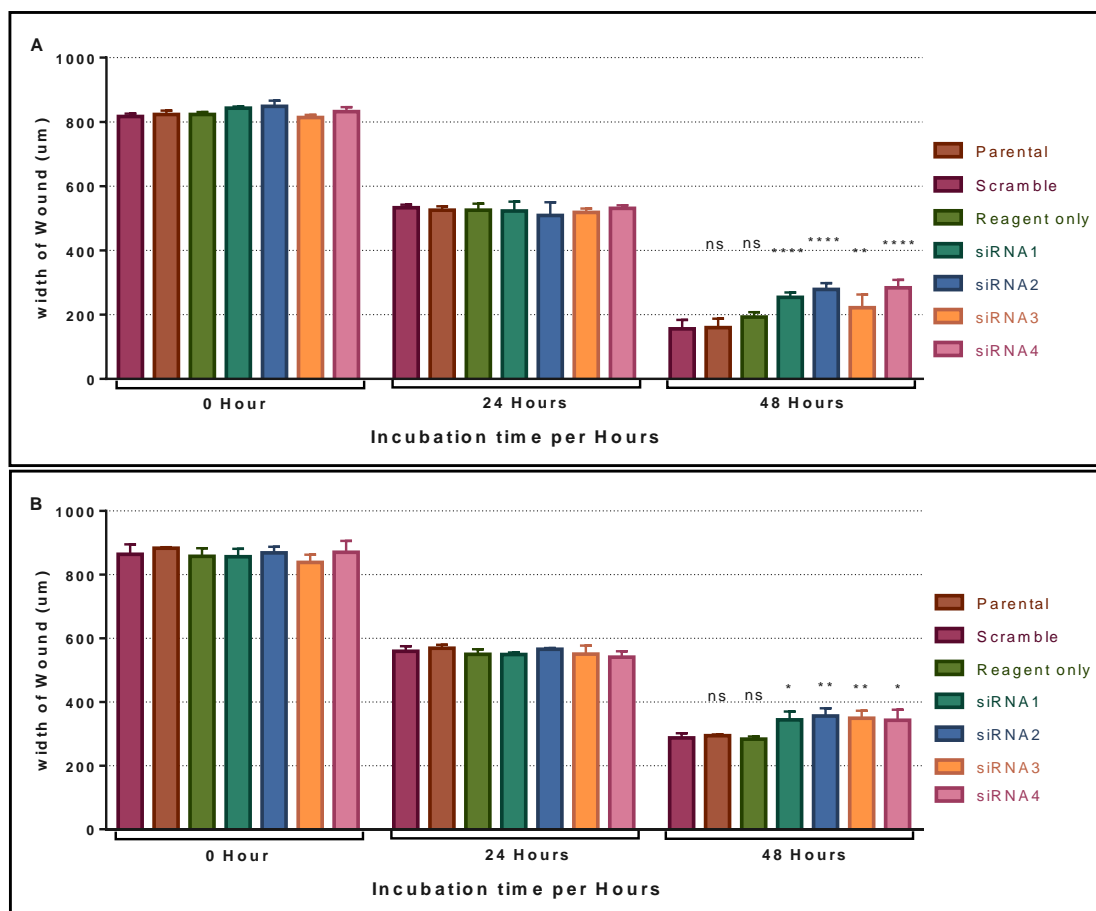
**Figure 5-13: In vitro scratch wound healing assay of TE-85 cells after silencing CTSZ expression**

Representative images of wound healing with control and different transfectants in 0, 24, 48 and 72 h after the transfection of siRNA TE-85 cells. Cells were wounded and monitored with microscope every 24 h. Images were captured using a 4x objective lens, were photographed with an Touptek ToupView and the width of the wound between the two edges was measured in triplicate using Image J software.

**Table 5-6: The impact of CTSZ silencing on migration rates of transfected Mg-63 and TE-85 at 48 h.**

	Dunnett's multiple comparisons test	Mean difference to the scrambled control (%)	P Value
<b>Mg-63</b>	Parental vs. Scrambled	2.56	0.9996
	Reagent only vs. Scrambled	23.53	0.1469
	siRNA1 vs. Scrambled	62.82	0.0001
	siRNA2 vs. Scrambled	78.40	0.0001
	siRNA3 vs. Scrambled	42.31	0.0017
	siRNA4 vs. Scrambled	81.86	0.0001
<b>TE-85</b>	Parental vs. Scrambled	2.54	0.9957
	Reagent only vs. Scrambled	-1.3	0.9997
	siRNA1 vs. Scrambled	19.76	0.0109
	siRNA2 vs. Scrambled	23.94	0.0015
	siRNA3 vs. Scrambled	21.60	0.0047
	siRNA4 vs. Scrambled	19.27	0.0135

The mean difference is the distance percentage change compared to the scrambled control and analysed using two-way ANOVA, (Dunnett's multiple comparisons test) to yield P values. Results were obtained from three separate experiments.



**Figure 5-14: The Effect of siRNAs Directed at CTSZ mRNA on the closure of the wound in a scratch wound healing assay using Mg-63 (A) or TE-85 (B) cells.**

The mean difference is the distance in micrometre change throughout 48 h. The bars show the width of the scratch width of the wound of parental, and Transfectant cells after 0, 24 h and 48h of incubation and by 72 h the closure of the wound was completely stop. Data are presented as mean  $\pm$  SD (n=3).

### 5.3. Discussion

CTSZ mRNA was found to be expressed in the cultured osteosarcoma cells and its functional tests were investigated *in vitro*. Mg-63, TE-85 and Saos-2 represent immature, moderately mature and mature osteoblast cells respectively (Pacheco-Pantoja et al., 2011). The RT-qPCR results indicate that CTSZ mRNA expression was inversely decreased with the maturation level of osteosarcoma cells. This suggest that CTSZ could be secreted from different cells (Kos et al., 2005, Nägler et al., 2004, Buhling et al., 2004, Wendt et al., 2007). The expression of CTSZ mRNA and protein were consistent with each other in Mg-63 and TE-85. However, in Saos-2 cells, CTSZ mRNA was only expressed at a low amount but its protein levels were not detectable by western blot (Figure 5-1). Whilst consistent in showing low level most likely reason for the difference is that the method for detection of mRNA (i.e. PCR) is much more sensitive than the method for detecting protein (i.e. Western blotting) (Koussounadis et al., 2015, Greenbaum et al., 2003)

The effect of suppressing CTSZ mRNA and protein was carried out in the Mg-63 and TE-85 cell lines using RNAi. The result showed that expression levels of CTSZ mRNA and protein was significantly reduced in the CTSZ-siRNAs transfected cells compare to scrambled control for both Mg-63 and TE-85. Therefore, taken together, the knockdown of CTSZ expression at the level of mRNA was generally correlated with a similar degree of knockdown of CTSZ protein as in agreement with previous studies (Abreu et al., 2009, Edfors et al., 2016)

As an initial stage to understanding the biological role of CTSZ in osteosarcoma cells, it was evaluated whether reduction in the level of CTSZ resulted in a reduction in



cell migration, invasion and proliferation of these cells. Overall, the proliferation, invasion and migration of cells were decreased with the reduced level of CTSZ. There was a clear and significant reduction of growth rate in CTSZ-siRNA transfectants cells compared to scramble control at day 6. Knockdown of CTSZ significantly reduced the number of cells invading through the Matrigel-coated membrane. Although there was no change in cell migration after 24 h, it was found that the wound closure rate was faster in the parental cells and scrambled control significant than in siRNA transfectants at 48 h, (Figure 5-14). Further statistical analysis was performed in order to assess the correlation between growth rate and migration/invasion. The rate of the growth in transfectant Mg-63 was significantly correlated with migration and invasion. Similarly, the proliferation of TE-85 transfectant showed a significantly correlation with migration and invasion ( $P= 0.009$  and  $0.024$  respectively). These results suggest that CTSZ is involved in all three cellular activities-invasion, migration and proliferation. Given that the observed reduction in invasion (24h), and migration (48h) precede that of proliferation (6 days), the finding indicate that CTSZ-siRNA-induced inhibition of invasion and migration is independent of its effect on the proliferation and the reduction in growth rate at 24 and 48 hours was insufficient to account for the reductions in migration/invasion observed (Tremel et al., 2009). Likewise, it is comprehensible to suggest that cellular migration may be a prerequisite for proliferation. Also. The blunted invasion is in agreement with the protease activity of CTSZ.

It was clear that there was a reduction of all function tests when CTSZ was suppressed using RNAi in Mg-63 and TE-85 cells. Nevertheless, Mg-63-CTSZ siRNA4 and TE-85-CTSZ-siRNA 2 showing the highest effect of the CTSZ suppression on the function tests. In other words, different siRNAs have efficiently on different cells. The

result here was consistent with other studies recently. Kraus et al indicated that suppression of CTSX induced cellular senescence and inhibited cell proliferation, and the cellular transmigration/ invasion in mouse embryonic fibroblasts (MEF) cells and neonatal normal human dermal fibroblasts (NHDF) (Kraus et al., 2011). In another study, antisense oligonucleotide suppression of CTSX protein revealed that cellular invasion of gastric adenocarcinoma cells infected with *H. pylori* was significantly lower compared with that in uninfected (Krueger et al., 2005). It was also reported that a lack of CTSX protein reduced the migration in neuroblastoma cells, and hence CTSX was suggested to play a role in migration of the SK-NBE2 cells (Gangoda et al., 2015). Recently, CTSZ gene was found to be estradiol-dependent, based on case-cohort genome-wide association study (GWAS) (Liu et al., 2014a). Furthermore, the suppression of CTSZ mRNA showed that the expression was changed in pathways associated with risk for bone fracture in postmenopausal breast cancer patients treated with aromatase inhibitors (AIs) for 5 years (Liu et al., 2014a). In concordance with the present experiment, overexpression of CTSZ in human hepatocellular carcinoma cell line (HCC cells) substantially enhanced the invasiveness, and significantly increased cell migration ability in a wound healing assay (Wang et al., 2011). Similarly, Jevnikar et al reported that overexpression of CTSX significantly raised transmigration and invasion of T lymphocytes compared with that of the wild-type cells through uncoated and ICAM1- coated polycarbonate membranes in a Transwell migration model. Matrigel-coated polycarbonate membrane. Therefore, they suggested that CTSX might be an important factor enabling T lymphocytes to function effectively as migratory cells during the immune response (Jevnikar et al., 2008)

Overall, the rate of expression of CTSZ is directly proportional to proliferation, invasion and migration of cells. Thus, these data suggest that CTSZ is involved in other cellular mechanism besides proteolysis. However, it is possible that proteolysis might play a role in proliferation, migration and invasion. CTSZ plays role in migration, invasion and proliferation of the osteosarcoma cells, Mg-63 and TE-85. This indicates that CTSZ may be a target protein that controls the migration, invasion and proliferation of bone cells. These results indicate that CTSZ may be an important molecular biomarker in early diagnosis and prediction of treatment response and prognosis of osteoporosis.

## **Chapter 6: Final Discussion and Future Work**

## 6.1 Final Discussion

Osteoporosis is a metabolic bone disorder characterized by low bone mass and increased incidences of fragility fracture (Ensrud and Crandall, 2017), particular in postmenopausal women where estrogen deficiency plays an important role in bone homeostasis (Feng and McDonald, 2011). The regulation of bone formation and bone resorption is mediated through estrogen receptors  $\alpha$  and/or  $\beta$  (Fitzpatrick, 2006). Many studies have previously reported the possible ways that estrogen could regulate genes in osteoblast cells (Krum et al., 2008, Kassem et al., 1996, Chen et al., 2002). However, since most of these studies were carried out using cultured cells or animal model systems (van Wijnen et al., 2013), the occurrence of estrogen deficiency as related to bone homeostasis in postmenopausal women is not well understood. Thus, it is important to investigate hormone and hormone responsive molecules associated with the development of osteoporosis in that population.

In this study, as summarized in Figure 6-1, a hormone responsive cell model system was established using osteosarcoma [osteoblast-like] cell lines, Mg-63, TE-85 and Saos-2 cells, where Mg-63 is identified as the cell line being the least mature, Saos-2 as the most differentiated and TE-85 may have a level of maturity somewhere between Mg-63 and Saos-2 (Pacheco-Pantoja et al., 2011). Both ER  $\alpha$  and  $\beta$  mRNAs and proteins were expressed in Mg-63 cells, which is in full agreement with previous reports (Lambertini et al., 2003, Solakidi et al., 2005, Kurosawa et al., 2002). However, the levels of ER  $\alpha$  proteins were much lower in both osteosarcoma cells, TE-85 and Saos2 cells, compared to that in Mg-63 cells. The ER  $\beta$  protein was detected in all three osteosarcoma cell lines and showed similar levels to those of MCF-7 cells. Thus, the

probable reason for fewer differentially expressed hormone-responsive genes being identified in TE85 and Saos2 cells is there being insufficient amount of ER  $\alpha$  proteins in both these cell lines for 17- $\beta$ -estradiol to form homo-dimers of ER $\alpha$  or hetero-dimers with ER $\beta$  to interact with specific DNA sequences to activate transcription (Khalid and Krum, 2016).

To assess the RNA Seq quality of each sample, mapping of the sequencing data was performed. The alignment of RNA-seq reads to the human reference genome showed that around 88 % of the total reads were identical to the human reference genome. The result here indicates good quality reads and the absence of contamination based on RNA-seq criteria. It has been reported that in a typical experiment, it is possible to align 60 -90 % of the reads to the reference genome, since accurate differential analysis depends on appropriately spliced read alignments which indicate good quality reads and the absence of contamination (Trapnell et al., 2012, Conesa et al., 2016).

A panel of differentially expressed hormone responsive genes were identified using RNA sequencing from established osteosarcoma cell line, Mg-63 cells after  $\beta$  estradiol treatment. 154 genes were up-regulated and 108 genes were down-regulated compared to non-treated controls. Some of these genes are known to be involved either in the regulation of bone remodelling, or in responding to estrogen. About 181 (65 %) of these genes were also significantly altered in the estrogen-treated MCF-7 cells. Therefore, these genes are suggested to be responsive to  $\beta$ -estradiol (Wang et al., 2007). The remaining 81 genes distinctly expressed in the Mg-63 cells, were not differentially expressed by  $\beta$ -estradiol in the MCF-7 cells, possibly reflecting the different origins of

the cells, i.e. bone and breast cells (Deroo et al., 2009) and suggests that these 81 genes might be bone-specific, estrogen-responsive genes/mRNAs.

A panel of 55 differentially-expressed hormone-responsive genes identified by RNA sequencing were validated initially using cultured osteosarcoma cells by RT-qPCR. The results showed that 45 out of 55 genes (around 82%) were detected at altered levels in the cultured osteosarcoma cells indicating that changes in levels of these mRNAs might be associated with the development of osteosarcoma cells.

It is noted that among identified estrogen responsive genes from this study, both CTSZ and TRAM2 genes were also reported to be associated with risk for bone fracture in a case-cohort genome-wide association study (GWAS) using samples from 1071 post-menopausal women patients treated with aromatase inhibitor (Liu et al., 2014a). This GWAS study confirms the relationship between estrogen and CTSZ and TRAM2 gene expression.

The estrogen receptor 1 gene (ESR1) and the RUNX2 gene, found differentially expressed in the present study, have also been identified in 3 different large international GWAS reports (Richards et al., 2008, Stykarsdottir et al., 2008, Estrada et al., 2012). One GWAS comprised 2094 UK and 6463 Western European subjects (Richards et al., 2008), a second GWAS comprised 10,026 Icelandic, 2269 Danish, and 1491 Australian subjects (Stykarsdottir et al., 2008), and a third GWAS comprised 80,000 BMD and 130,000 fracture cases and controls of European and east Asian origin (Estrada et al., 2012). This suggests that ESR1 and RUNX2 are widely associated with risk factors for osteoporosis. Although the remaining genes identified from this study have not been reported in GWAS, this could be due to the natural complexity of osteoporosis, variations in hormone levels, fracture history, and the heterogeneity of different

populations, age and ethnicity of the subjects of the studies (Mendoza et al., 2012). Furthermore, GWAS analyses identify genome variations, but not gene expression levels (Liu et al., 2014a), whereas the present study focuses directly on expressed mRNA levels.

Upon further analysis, it was revealed that some of the validated genes were associated with the development of osteoporosis in clinical samples, PBMCs, using RT-qPCR. The levels of ANXA1, S100A4, and XBP1 mRNAs were significantly decreased in PBMCs associated with a reduced T-score in osteoporotic patients ( $p = 0.0291$ ,  $0.00034$ ,  $0.0001$ , respectively). Particularly, it was found previously that suppression of ANXA1 decreases the formation of osteoclast in both arthritis and monocyte-derived human macrophages by promoting IL-6 release (Kao et al., 2014). Also, ANXA1 has been shown to be a bone formation modulator in rat osteoblasts in primary cell cultures through enhancing PTH-mediated cAMP stimulation by binding calcium ions that act as a second messenger relaying the PTH signal intracellularly (Suarez et al., 1993). Potential target miRNAs of ANXA1 mRNA were identified using 4 different algorithms (miRanda, miRDB, miRWalk and Targetscan) within the miRWalk 2.0 database. miR100-5p and miR-196b-3p showed to be target miRNAs for ANXA1. In PBMCs, ANXA1 mRNA was positively regulated by miR100-5p ( $p = 0.0002$ ), and negatively regulated by miR196b-3p ( $p < 0.0001$ ). ANXA1 has previously been identified as a target for regulatory miRNAs 196a and 196b, with inversely proportional expression levels detected in a variety of cancer cell lines (Luthra et al., 2008). Also, Gir *et al* (2012) and Kim *et al* (2009) showed that miRNA 196a inversely regulates the osteoblast precursor (Gir et al., 2012, Kim et al., 2009). This is in concordance with the reported

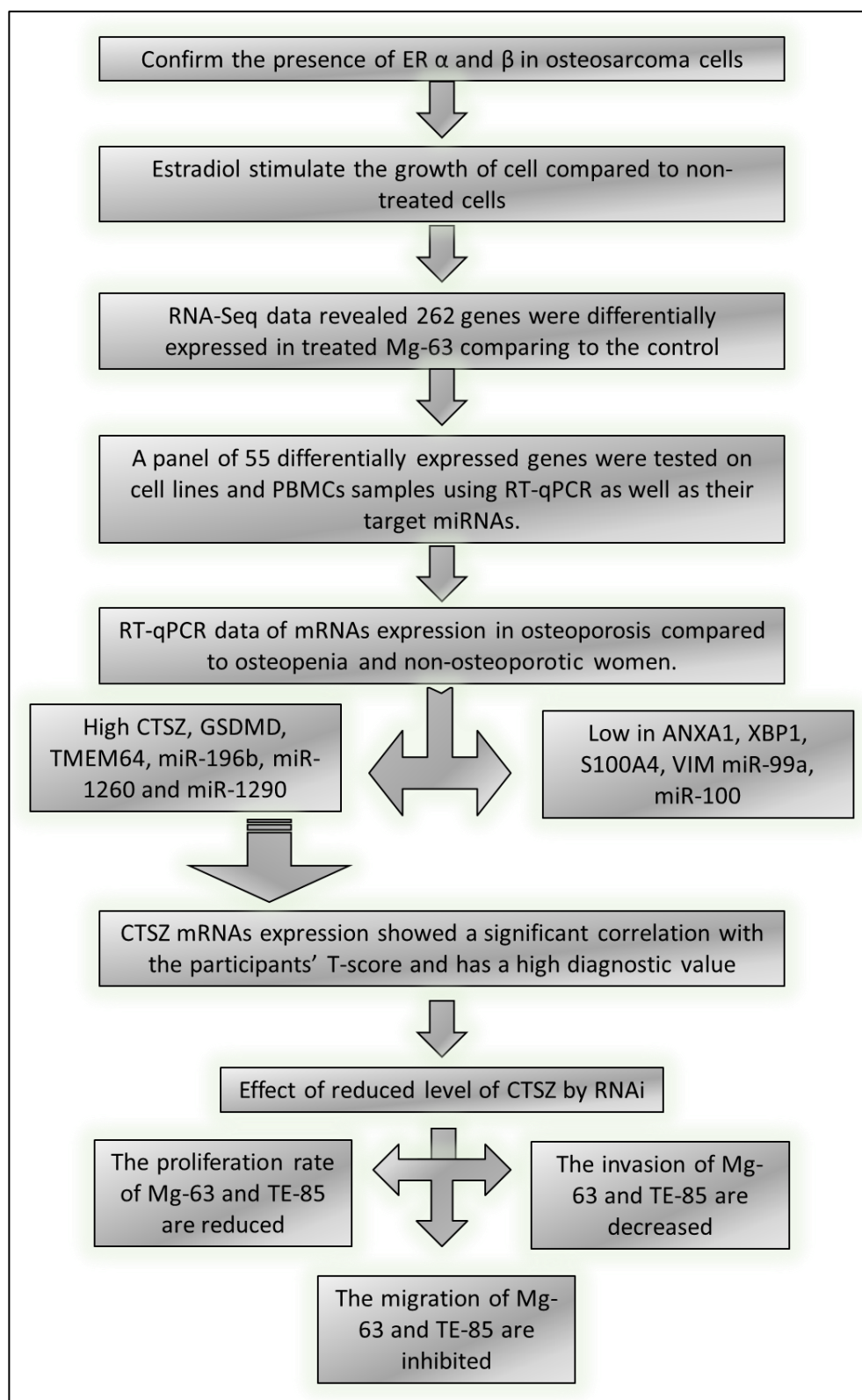


role of ANXA1 favoring osteoclast formation, evident by the reduced expression of ANXA1 in postmenopausal osteoporosis compared to non-osteoporotic participants’.

Cathepsins are a family of serine, cysteine, and aspartyl protease enzymes involved in cell turnover (Santamaria et al., 1998). Members of the Cathepsin family have been found to be involved in serious clinical conditions including cancer, neurodegenerative disorders, and arthritis (Santamaria et al., 1998, Mohamed and Sloane, 2006), and the association of cathepsins with bone remodelling in osteoclasts is well established (Holzer et al., 2005, Lang et al., 2004, Han et al., 2009, Wilson et al., 2009). However, the role of CTSZ in bone disorder has not been sufficiently investigated until Liu *et al* confirm the presence of CTSZ to be estradiol-dependent, based on case-cohort GWAS and suggested that suppression of CTSZ mRNA result in changing in pathways associated with risk for bone fracture in postmenopausal breast cancer patients (Liu et al., 2014a). Our data indicate that the expression of CTSZ mRNA showed an inverse relationship to the maturity degree of osteosarcoma cells. Also, the levels of CTSZ mRNA were much higher in PBMC of osteoporotic patients compared with osteopenia patients and non-osteoporotic controls ( $p = 0.0007$ ). Increasing levels of CTSZ mRNA in PBMC significantly correlated with the reduced bone mineral density *T*-score in osteoporosis patients ( $p = 0.0002$ ). ROC analysis for CTSZ mRNA showed an AUC of 0.806 and  $p = 0.002$ , suggesting that CTSZ mRNA might have utility for further development as a biomarker for osteoporosis in post-menopausal women. Furthermore, through a follow-up cellular functional study, suppression of CTSZ mRNA/protein expression using RNAi significantly inhibited invasion, migration and proliferation of Mg-63 and TE-85 cells. Kraus et al indicated that suppression of CTSZ inhibited cell proliferation, and the cellular transmigration/ invasion in mouse embryonic

fibroblasts (MEF) cells and neonatal normal human dermal fibroblasts (NHDF) (Kraus et al., 2011). Also, in concordance with the present experiment, it was found that overexpression of CTSZ in human hepatocellular carcinoma cell line (HCC cells) and T lymphocytes significantly raised the transmigration and invasion compared with that of control cells (Wang et al., 2011, Jevnikar et al., 2008)

Taken together, these results suggest that CTSZ might be a target protein that modulates the migration, invasion and proliferation of bone cells. To our knowledge, this is the first report of the presence of CTSZ mRNA and protein in cultured osteosarcoma cells, and the first demonstration of an association between CTSZ and osteoporosis in clinical samples. The elevated CTSZ mRNA expression level, as detected in low BMD participants, probably stimulate more PBMC, osteoclast precursors, migration/invasion through blood vessel walls to bone resorption surface in vivo. This is where they differentiate into higher number of mature osteoclasts eventually leading to enhanced bone resorption (i.e. osteoporosis). These findings indicate that CTSZ may be an important molecular biomarker for both the in early diagnosis of osteoporosis as well as prediction of treatment response and prognosis.



**Figure 6-01: Project summary and findings.**

## 6.2 Future study

To fully establish this study following further studies are needed:

- Although there are many pathways through which osteoclast can be activated, the results presented in this thesis clearly indicate CTSZ is a major inducer of bone loss in osteoporosis in post-menopausal women. Nevertheless, further larger studies will be required to identify the exact pathways and targets, such as Wnt signalling pathway through which inhibiting CTSZ with the biologic agents could bring about such outcome.
- Some in-vivo studies are needed to assess the ability of suppressed CTSZ to reduce the migration and invasion abilities of osteoclast cells and the ability of PBMCs to form osteoclast cells and the possibility of using it as a treatment target.
- Other identified estrogen-responsive genes, such as ANXA1, GSDMD, TMEM64, miR-196b-3p and miR-100-5p, need to be tested with large number of clinical samples to have a clear picture about molecular mechanism underlying estrogen deficiency in postmenopausal osteoporosis women
- As part of the national research programme in the Kingdom of Saudi Arabia, part of the current work will be expanded to include osteoporosis in postmenopausal women patients from that country since this disease has a high prevalence in that area with, potentially, additional more risk factors, particularly lifestyle.

## **Chapter 7: References**

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## Appendix

## 8.1 Laboratory Equipment

**Table 8-1 Equipment used in this project**

No.	Equipment	Manufacture and Model
1	-80° Freezer	ESCO Lexicon ULT Freezer
2	Automated Cell counter	Scepter™, MILLPORE, UK
3	BIO-RAD T100 Thermocycler.	Bio-Rad Laboratories Ltd., UK
4	BioRad ChemiDOCTM digital imager	Bio-Rad Laboratories Ltd., UK
5	CryoPure Tube 1.8 mL	STARLAB Ltd, UK
6	ESCO Class II BS Cabinet AC2-4G1SP Biological safety cabinet class II.	ESCO, UK
7	Gel power pack Power Pac™ HC,	BIO-RAD, UK
8	Gel Transfer Trans-Blot®Turbo Transfer System,	BIO-RAD, UK
9	Genies2 Vortex mixer	Scientific Laboratory Supplies Ltd
10	Microcentrifuge SCF2	Fisher Scientific UK
11	Nikon Diaphot inverted microscope/D50 camera	Nikon
12	PCR hood	PCR Workstation
13	Qubit® Protein Assay Kit using the Qubit® Flurometer 2.0	Invitrogen Ltd, Paisely, UK
14	Roche Lightcycler® 96 Real-Time PCR System.	Roche Diagnostics Ltd, UK
15	SPECTROstar Nano	LabTech, Germany
16	Thermo Heraeus Megafuge 16R Centrifuge (Refrigerated)	ThermoFisher, UK
17	Thermo Scientific Heraeus Fresco Centrifuge 17R (Refrigerated)	ThermoFisher Ltd, UK
18	Thermo Scientific NanoDrop™ 2000 spectrophotometer	ThermoFisher Ltd, UK
19	ThermoBloc, FALC	Lurano, ITALY
20	Waterbath	Grant, JB Aqua 18 Plus
21	-80° Freezer	ESCO Lexicon ULT Freezer

**Table 8-2 Consumables used in this study**

<b>No.</b>	<b>Material</b>	<b>Manufacture and Model</b>
<b>1</b>	1.5mL ultra High Recovery (UHR) Microcentrifuge Tube	STARLAB Ltd, UK
<b>2</b>	2.0mL ultra High Recovery (UHR) Microcentrifuge Tube	STARLAB Ltd, UK
<b>3</b>	6/12/24 well tissue culture plates	Techno Plastic Products
<b>4</b>	96-well real time PCR plates	Starlab
<b>5</b>	CryoPure Tube 1.8 mL	STARLAB Ltd, UK
<b>6</b>	Disposable serological pipette tips	Starlab
<b>7</b>	Filter Tip (Sterile), 0.5-10µl	Fisher Scientific Ltd, UK
<b>8</b>	Filter Tip (Sterile), 2-20µl	Fisher Scientific Ltd, UK
<b>9</b>	Filter Tip (Sterile), 100-1000µl	Fisher Scientific Ltd, UK
<b>10</b>	Greiner Bio-One 10 mL Leucosep® tube, sterile for 3-8 mL blood	Greiner bio-one Ltd, UK
<b>11</b>	SARSTEDT S-Monovette® 9mL K3E, Potassium EDTA	STARLAB Ltd, UK
<b>12</b>	STARLAB 15 mL Polypropylene Centrifuge Tubes	STARLAB Ltd, UK
<b>13</b>	STARLAB 50 mL Polypropylene Centrifuge Tubes	STARLAB Ltd, UK
<b>14</b>	TC-25, TC-75 flasks	STARLAB Ltd, UK
<b>15</b>	Filtropur S 0.45 syringe filter	SARSTEDT, UK



## 8.2 Materials for Blood Collection and Molecular Biology

**Table 8-3 Chemical and reagent used in this project**

No.	Chemical and Reagent	Manufacture
1	Charcoal Stripped Fetal Bovine Serum,	Sigma-Aldrich Company Ltd., UK
2	Chloroform [Sigma] (>99.5% Purity, 119.38 g/mol (mW))	Sigma-Aldrich Company Ltd., UK
3	Dimethyl sulfoxide (DMSO),	Sigma-Aldrich Company Ltd., UK
4	Dulbecco's Modified Eagle's Medium,	Sigma-Aldrich Company Ltd., UK
5	Ethyl alcohol, Pure $\geq 95\%$ [Sigma]	Sigma-Aldrich Company Ltd., UK
6	Fetal Bovine Serum (FBS),	Sigma-Aldrich Company Ltd., UK
7	Ficoll-Paque Premium 1.077g/ml	GE Healthcare Life Sciences, UK
8	HiPerFect® Transfection Reagent	QIAGEN Ltd, UK.
9	L-Glutamine solution,	Sigma-Aldrich Company Ltd., UK
10	MEM Non-essential Amino Acid Solution (100×),	Sigma-Aldrich Company Ltd., UK
11	Methanol	Sigma-Aldrich Company Ltd., UK
12	miScript II RT Kit	QIAGEN Ltd, UK.
13	miScript SYBR Green PCR Kit	QIAGEN Ltd, UK.
14	Penicillin-Streptomycin,	Sigma-Aldrich Company Ltd., UK
15	Pure-ethanol molecular biology grade	Sigma-Aldrich Company Ltd., UK
16	PureLink® RNA Mini Kit	Ambion by life technology, UK
17	Qubit® Protein Assay Kit	Ambion by life technology, UK
18	RIPA Lysis and Extraction Buffer	ThermoFisher, UK
19	RNaseZap® Solution	Life Technologies Ltd, UK
20	Roswell Park Memorial Institute (RPMI) without L-glutamine	Sigma-Aldrich Company Ltd., UK
21	RT2 First Strand Kit	QIAGEN Ltd, UK.
22	RT <sup>2</sup> SYBR Green qPCR Mastermix	QIAGEN Ltd, UK.
23	Shandon™ Kwik-Diff™ Stains	ThermoFisher, UK
24	SuperSignal™ West Femto Chemiluminescent Substrate	ThermoFisher, UK
25	Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich Company Ltd., UK
26	Trizol® Reagent	Life Technologies Ltd, UK
27	Trypsin-EDTA solution	Sigma-Aldrich Company Ltd., UK
28	$\beta$ -Mercaptoethanol	BIO-RAD, UK

## 8.3 Gene Ontology Reference

**Table 8-4: ontology reference of identified genes in Mg-63 using cufflinks software by Earlham Institute**

GO class	Genes	Reference	Evidence
Androgen metabolic process	ESR1	GO_REF:0000107	IEA
Angiotensin maturation	CTSZ	Reactome:R-HSA-2022381	TAS
Bone morphogenesis	MEF2CA	ZFIN:ZDB-PUB-030304-18 PMID:12588850	IMP
Bone trabecula formation	COL1A1	GO_REF:0000107	IEA
	MMP2	GO_REF:0000107	IEA
Cartilage development involved in endochondral bone morphogenesis	COL1A1	GO_REF:0000107	IEA
Cellular response to estradiol stimulus	ESR1	RGD:1600115	IEA
	MMP2	RGD:2325711 PMID:19861308	IMP
	UGT1A1	GO_REF:0000107	IEA
Collagen fibril organization	COL14A1	PMID:2187872	NAS
	COL1A1	GO_REF:0000107	IEA
	COL1A2	GO_REF:0000107	IEA
	COL3A1	GO_REF:0000107	IEA
Embryonic cranial skeleton morphogenesis	PDGFRA	GO_REF:0000107	IEA
Embryonic skeletal system morphogenesis	HOXB3	GO_REF:0000107	IEA
	HOXB4	RGD:1600115	IEA
	HOXB6	GO_REF:0000107	IEA
	MEF2C	MGI:MGI:3714538 PMID:17420000	IMP
	ZEB1	GO_REF:0000107	IEA
Endochondral ossification	COL1A1	GO_REF:0000107	IEA
	MEF2C	RGD:1600115	IEA
Estrogen metabolic process	PDGFRA	GO_REF:0000107	IEA
	UGT1A1	PMID:8780690	TAS
Growth hormone secretion	LTBP4	PMID:9660815	TAS
Insulin secretion	ANXA1	GO_REF:0000107	IEA
Muscle contraction	CALD1	Reactome:R-HSA-445355	TAS
	MYOM2	PMID:7505783	TAS
	TPM2	Reactome:R-HSA-445355	TAS
	VIPR1	PMID:8390245	TAS
Muscle filament sliding	TPM2	Reactome:R-HSA-390522	TAS
Negative regulation of cartilage condensation	MMP2	PMID:17604018	IMP
Negative regulation of hormone biosynthetic process	CACNA1A	GO_REF:0000107	IEA
Positive regulation of gene expression	ESR1	PMID:21047992	IDA

<b>Positive regulation of parathyroid hormone secretion</b>	<b>FGFR1</b>	GO_REF:0000107	IEA
<b>Positive regulation of steroid hormone biosynthetic process</b>	<b>IGF2</b>	MGI:MGI:4417868 GO_REF:0000096	ISO
<b>Prolactin secretion</b>	<b>ANXA1</b>	GO_REF:0000107	IEA
<b>Regulation of aldosterone metabolic process</b>	<b>KCNMA1</b>	PMID:8973172	ISA
<b>Regulation of cardiac muscle cell contraction</b>	<b>PDE4D</b>	GO_REF:0000024	ISS
<b>Regulation of cardiac muscle contraction by regulation of the release of sequestered calcium ion</b>	<b>GSTM2</b>	PMID:17023043	IC
<b>Regulation of hormone secretion</b>	<b>ANXA1</b>	PMID:19625660	IMP
<b>Regulation of insulin secretion</b>	<b>CACNA1A</b>	Reactome:R-HSA-422356	TAS
<b>Regulation of skeletal muscle contraction by regulation of release of sequestered calcium ion</b>	<b>GSTM2</b>	PMID:17023043	IC
<b>Relaxation of cardiac muscle</b>	<b>GSTM2</b>	PMID:21323602	TAS
<b>Response to estradiol</b>	<b>ALDH1A2</b>	GO_REF:0000107	IEA
	<b>ANXA1</b>	GO_REF:0000107	IEA
	<b>COL1A1</b>	GO_REF:0000107	IEA
	<b>ESR1</b>	RGD:2313676 PMID:9528993	IEP
	<b>IFI27</b>	RGD:68731 PMID:11356686	IEP
	<b>IGF2</b>	RGD:2311518 PMID:18946176	IEP
	<b>PDGFRA</b>	RGD:2292171 PMID:14724432	IEP
<b>Retinoic acid biosynthetic process</b>	<b>ALDH1A2</b>	MGI:MGI:4417868 GO_REF:0000096	ISO
<b>Retinoic acid metabolic process</b>	<b>ALDH1A2</b>	GO_REF:0000024	ISS
	<b>UGT1A1</b>	PMID:20308471	IC
	<b>UGT1A3</b>	PMID:20308471	IC
	<b>UGT1A7</b>	PMID:20308471	IC
	<b>UGT1A8</b>	PMID:20308471	IC
	<b>UGT1A9</b>	PMID:20308471	IC
<b>Retinol metabolic process</b>	<b>ALDH1A2</b>	GO_REF:0000041	IEA
<b>Skeletal muscle contraction</b>	<b>CCDC78</b>	PMID:22818856	IMP
<b>Skeletal system morphogenesis</b>	<b>COL1A1</b>	MGI:MGI:3710444 PMID:17440987	IGI
	<b>FGFR1</b>	PMID:15863030	TAS
	<b>HOXB4</b>	RGD:1624291	ISO
	<b>MMP2</b>	RGD:1624291	ISO
	<b>PDGFRA</b>	RGD:1624291	ISO
<b>Smooth muscle contraction</b>	<b>PDE4D</b>	GO_REF:0000107	IEA
<b>Smooth muscle contraction involved in micturition</b>	<b>KCNMA1</b>	PMID:11641143	IDA
<b>Striated muscle contraction</b>	<b>DTNA</b>	PMID:10767327	TAS
	<b>MYOM2</b>	GO_REF:0000033	IBA
<b>Response to estrogen</b>	<b>ESR1</b>	PMID:11581164	IDA
	<b>PDGFRA</b>	<b>RGD:2292171 PMID:14724432</b>	<b>IEP</b>
	<b>KRT19</b>	GO_REF:0000107	IEA

<b>Cellular response to estrogen stimulus</b>	<b>ESR1</b>	<b>GO_REF:0000107</b>	<b>IEA</b>
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